Migrant or resident? The identification of group 1 innate lymphoid cells in the murine central nervous system

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Abstract

Innate lymphoid cells (ILCs) are tissue resident cells that play important roles in the maintenance of tissue homeostasis and the regulation of inflammation. ILCs have been extensively characterized in various organs such as the gut and the liver, revealing its diversity and tissue-specific specialization. However, whether ILCs are present in the central nervous system (CNS) and if so, what are their phenotype and function in this unique organ are questions that remain unanswered. NK cells are the longer-known and better-studied ILC members that share many phenotypical and functional features with ILC1s, making the distinction between these two subsets difficult. In particular, both mouse NK cells and ILC1s express NK1.1 and NKp46, secrete IFN-γ and contribute in the host defense against tumors and intracellular infections. In the context of autoimmunity, NK cells have been shown to display immunomodulatory roles, particularly in multiple sclerosis (MS), the most common inflammatory autoimmune disease of the CNS. Using the animal model of MS, the experimental autoimmune encephalomyelitis (EAE), our group showed that protective mature NK cells are recruited to the CNS on an CX3CR1-dependent manner. Based on that observations, my PhD project aimed to 1) define the chemokine receptors that mediate the recruitment of the immature NK cells into the inflamed CNS and to 2) determine whether the phenotypically defined NK cells (CD3-NK1.1+ cells) that are present in the CNS during steady state constitute *bona fide* NK cells or constitute also other group 1 ILC subsets.

The results of the present work indicate that the CD3-NK1.1+ cells present in the healthy CNS comprise diverse group 1 ILC subsets that include the conventional NK cells, ILC1s, intermediate-ILC1s and ex-ILC3s. CXCR3 was expressed on ILC1s and a fraction of immature NK cells, but did not contributed to the recruitment of NK cells into the CNS in the EAE model. Instead, CXCR3 was downregulated in NK cells and ILC1s during inflammation, indicating a ligand induced internalization. Furthermore, the phenotypic and functional characterization of the newly identified CNS-ILC1s substantiated their identity as a separate subset from conventional NK cells. The exclusive expression of CD49a, CD69, CXCR6, DNAM-1^{high}, TRAIL and CD200R, and lack of Eomes distinguished the ILC1s from the NK cells in the CNS. In addition, ILC1s secreted IFN-γ and more TNF-α than NK cells upon stimulation in the healthy and EAE mice. ILC1s were the dominant group 1 ILC subset in the choroid plexus and brain parenchyma and were also present in the meninges. During EAE, in addition to CXCR3, the expression of TRAIL was downmodulated, and an increase in the numbers of all group 1 ILCs was observed in the brain parenchyma. However, contrary to NK cells, ILC1s did not proliferate during EAE suggesting an *in situ* NK cell to ILC1 differentiation. In sum, the present work provides for the first time a comprehensive characterization of group 1 ILCs in the CNS, which reveals a possible gatekeeper and neuroprotective role of ILC1s in this unique organ.

Zussamenfassung

Angeborene lymphoide Zellen (ILCs) sind sich im Gewebe befindliche Zellen, die eine wichtige Rolle bei der Aufrechterhaltung der Gewebehomöostase und der Regulation von Entzündungen spielen. ILCs wurden in verschiedenen Organen wie dem Darm und der Leber umfassend untersucht und zeigten eine hohe Diversität und gewebespezifische Differenzierung. Ob ILCs im zentralen Nervensystem (ZNS) vorhanden sind und wenn ja, welchen Phänotyp und welche funktionellen Eigenschaften sie in diesem einzigartigen Organ aufweisen, sind Fragen, die bisher unbeantwortet blieben. NK-Zellen sind die seit langem bekannten und besser untersuchten ILC-Mitglieder, die viele phänotypische und funktionelle Merkmale mit ILC1s teilen. Es macht die Unterscheidung zwischen diesen beiden Untergruppen schwierig. Sowohl Maus-NK-Zellen als auch Maus-ILC1s exprimieren NK1.1 und NKp46. Beide sezernieren IFN-γ und tragen zur Abwehr von Wirten gegen Tumore und intrazelluläre Infektionen bei. Im Zusammenhang mit der Autoimmunität wurde gezeigt, dass NK-Zellen eine immunmodulatorische Rollen spielen. Dies wird besonders bei Multipler Sklerose (MS), der häufigsten entzündlichen Autoimmunerkrankung des ZNS, klar. Anhand des Tiermodells von MS, der experimentellen autoimmunen Enzephalomyelitis (EAE), zeigte unsere Gruppe, dass reife NK-Schutzzellen auf CX3CR1-abhängige Weise in das ZNS rekrutiert werden. Auf der Grundlage dieser Beobachtungen will ich in meinem PhD Projekt die Chemokinrezeptoren definieren, die die Rekrutierung der unreifen NK-Zellen in das entzündete ZNS vermitteln. Des Weiteren will ich herausfinden, ob die phänotypisch definierten NK-Zellen (CD3-NK1.1 + -Zellen) im gesunden ZNS vorhanden sind und ob dies echte NK-Zellen sind, oder sie zu den ILC1s gezählt werden können.

Die Ergebnisse der vorliegenden Arbeit zeigen, dass die im gesunden ZNS vorhandenen CD3-NK1.1+ -Zellen verschiedene Typ-1-ILC-Subsets umfassen, die die herkömmlichen NK-Zellen, ILC1s, Intermediat-ILC1s und Ex-ILC3s umfassen. CXCR3 wurde auf ILC1s und einer Fraktion von unreifen NK-Zellen exprimiert, trug jedoch nicht zur Rekrutierung von NK-Zellen in das ZNS im EAE-Modell bei. Stattdessen wurde CXCR3 während der Entzündung in NK-Zellen und ILC1-Zellen herunterreguliert. Was auf eine, durch den Liganden induzierte, Internalisierung hindeutet. Die phänotypische und funktionelle Charakterisierung der neu identifizierten ZNS-ILC1s untermauert ihre Identität als separate Untergruppe von herkömmlichen NK-Zellen. Die ausschließliche Expression von CD49a, CD69, CXCR6, DNAM-1high, TRAIL und CD200R und das Fehlen von Eomes unterschieden die ILC1 von den NK-Zellen im ZNS. Zusätzlich sezernierten ILC1s bei Stimulation in den gesunden und EAE-Mäusen IFN-γ und mehr TNF-α als NK-Zellen. ILC1s waren die dominante Typ-1-ILC Subgruppe im Plexus choroideus und im Gehirnparenchym und waren auch in den Meningen

vorhanden. Während der EAE wurde zusätzlich zu CXCR3 die Expression von TRAIL herunterreguliert, und im Gehirnparenchym wurde ein Anstieg der Anzahl aller Typ-1-ILCs beobachtet. Im Gegensatz zu NK-Zellen proliferierten ILC1s jedoch nicht während der EAE, was eine In-situ-NK-Zelle für die ILC1-Differenzierung vermuten lässt. Zusammenfassend bietet die vorliegende Arbeit zum ersten Mal eine umfassende Charakterisierung von ILCs des Typs I im ZNS, die eine mögliche *Gatekeeper* und neuroprotektive Rolle von ILC1s in diesem einzigartigen Organ aufzeigen könnte.

Table of Contents

1 List of abbreviations 11
2 List of figures 14
3 Introduction 15
3.1 Overview of the immune system and its innate and adaptive arms 15
3.2 Innate lymphoid cells, the counterpart of T lymphocytes 17
3.2.1 NK cells 19
3.2.2 ILC1s 21
3.2.3 ILC2s 22
3.2.4 ILC3s 22
3.2.5 LTi cells 23
3.2.6 ILC plasticity and heterogeneity 24
3.3 Tissue residency, migration and circulation of ILCs 25
3.3.1 Chemokines and adhesion molecules 26
3.3.2 Homing of immune cells into the healthy and inflamed CNS 27
3.4 Multiple sclerosis 30
3.4.1 Experimental autoimmune encephalomyelitis as the mouse model of MS 32
3.4.2 The role of group 1 ILCs in MS and EAE 33
4 Aims of the thesis 37
5 Materials and Methods 38
5.1 Laboratory materials 38
5.1.1 Buffers, solutions and cell culture media 38
5.1.2 Additional chemicals and reagents 40
5.1.3 Kits 40
5.1.4 Devices 41
5.1.5 Software 41
5.1.6 General consumables 42
5.2 Mice 43
5.3 *In vivo* methods 43
5.3.1 Induction and assessment of active EAE 43
5.3.2 Anti-CXCR3 antibody treatment 43
5.3.3 Mouse perfusion and organ removal 44
5.4 Isolation of immune cells from mouse tissue 44
5.4.1 Blood 44
5.4.2 Spleen 44
5.4.3 Lymph nodes 45
5.4.4 Liver 45
5.4.5 Central nervous system 46

5.4.6 Meninges 46
5.4.7 Choroid plexus 47
5.5 Cell sorting methods....47
5.5.1 Magnetic activated cell sorting 47
5.5.2 Flow activated cell sorting 48
5.6 *In vitro* assays 48
5.6.1 Cell culture 48
5.6.2 Cell counting 48
5.6.3 Stimulation of immune cells....49
5.7 Flow cytometry 49
5.7.1 List of antibodies and reagents used for flow cytometry 50
5.7.2 Staining of cells for flow cytometry 52
5.8 Statistics....52
6 Results 53
6.1 Characterization of the chemokine receptors of immature NK cells in the CNS 53
6.1.1 CX3CR1 mediate the recruitment of mature NK cells to the CNS during neuroinflammation 53
6.1.2 Immature NK cells are present in the healthy and inflamed murine CNS....54
6.1.3 Immature NK cells in the CNS express CXCR3....56
6.1.4 CXCR3+ NK cells decrease in the CNS during EAE....57
6.1.5 The surface expression of CXCR3 on NK cells decreases after activation with CXCL10....57
6.1.6 Activation of CXCR3+ NK cells with CXCL10 does not induce NK cell maturation nor IFN-γ secretion 58
6.1.7 Anti-CXCR3 antibody treatment does not alter the proportion of immature NK cells in the CNS of EAE mice....59
6.2 Identification of different ILC group 1 subsets in the CNS....61
6.2.1 Immature NK cells express the ILC1 marker CD49a 61
6.2.2 CD3-NK1.1+ CNS cells comprise diverse ILC populations 62
6.2.3 NK cells present in the CNS have a mature phenotype, ILC1s are CD11b^{low} and CD27+……....64
6.2.4 ILC1s of the CNS are characterized by the expression of CXCR6, TRAIL and DNAM-1....64
6.2.5 Besides CXCR3, ILC1s modulate TRAIL expression....65
6.2.6 ILC1s express more TNF-α than NK cells in the healthy and inflamed CNS....66
6.2.7 Group 1 ILCs are differentially distributed within the CNS compartments and are increased in the brain parenchyma during EAE 68
6.2.8 NK cells, but not ILC1s, proliferate in the CNS during EAE 69

7 Discussion 71
7.1 Characterization of the chemokine tools of immature NK cells in the CNS and periphery 71
7.2 Identification of different ILC group 1 subsets in the CNS 75
7.3 Graphical summary 82
7.4 Outlook 83
8 References 85
9 Selbständingkeitserklärung 104

1 List of abbreviations

Abbreviation	Full name
7-AAD	7-aminoactinomycin
ADCC	antibody dependent cell cytotoxicity
AF	alexa fluor
APC (fluorophore)	allophycocyanin
APC (immunity)	antigen presenting cell
BBB	blood brain barrier
BM	bone marrow
BrefA	brefeldine A
BSA	bovine serum albumin
BV	brilliant violet
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CFA	complete Freund’s adjuvant
CMV	cytomegalovirus
CNS	central nervous system
CP	choroid plexus
CSF	cerebrospinal fluid
CX3CL	CX3C chemokine ligand
CX3CR	CX3C chemokine receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DNA	deoxyribonucleic acid
DC	dendritic cell
DNAM-1	DNAX accessory molecule I
DNase	deoxyribonuclease
EAE	experimental autoimmune encephalomyelitis
EDSS	expanded disability status scale
Eomes	eomesodermin
FACS	flow activated cell sorting
Fc	constant fraction
FcR	constant fraction receptor
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
FSC	forward scattered light
g	relative centrifuge force (g = 9.81 m/s2)
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
intILC	intermediate innate lymphoid cell
KIR	killer cell immunoglobulin-like receptor
LN	lymph nodes
LTi	lymphoid tissue inducer
MACS	magnetic activated cell sorting
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NCR	natural cytotoxicity receptor
Nfil3	nuclear factor, interleukin 3 regulated
NK	natural killer
NKG2	killer cell lectin-like receptor subfamily K
NKp	natural killer cell p-related protein
NSC	neural stem cell
p.i.	post immunization
PacBlue	pacific blue
PBS	phosphate buffered saline
PE	phycoerythrin
PE Cy7	phycoerythrin cyanine 7 conjugate
PerCPCy5	peridinin chlorophil protein complex cyanine 5
PFA	conjugate
PLP	paraformaldehyde
PMA	myelin proteolypic protein
p-value	phorbol 12-myristate 13-acetate

RFP	probability value
RNA	red fluorescent protein
ROS	ribonucleic acid
rpm	reactive oxygen species
RRMS	revolutions per minute
RT	relapsing remitting multiple sclerosis
S1P	room temperature
S1P1	sphingosine-1-phosphate
SEM	sphingosine-1-phosphate receptor 1
SJL	standard error of the mean
SSC	Swiss Jim Lambert
SVZ	sideward scattered light
T-bet	subventricular zone
TGF	t-box transcription factor TBX21
Th	transforming growth factor
TNF	T helper cell
TRAIL	tumor necrosis factor
Treg	TNF-related apoptosis-inducing ligand
WT	regulatory T cell
	wild type

2 List of figures

Introduction

Figure I. Development and function of ILCs……..…18

Figure II. Plasticity of NK cells, ILC1s and ILC3s……..…...24

Results

Figure 1. CX3CR1-dependent recruitment of mature NK cells into de inflamed CNS……53

Figure 2. NK cell maturation subsets in the healthy and inflamed CNS……55

Figure 3. Chemokine receptors on immature NK cells in the CNS and the periphery……..…56

Figure 4. Expression of chemokine receptors on NK cells during EAE……..57

Figure 5. Surface expression of CXCR3 after CXCL10 ligation……..……...58

Figure 6. Maturation of NK cells after short and long term incubation with CXCL10…….…...59

Figure 7. Effect of anti-CXCR3 treatment during EAE on the recruitment of NK cells into the CNS and periphery ……..…….60

Figure 8. Expression of CD49a and CD49b in the NK cell maturation subsets……...61

Figure 9. Identity of the NK1.1+ CNS populations defined by the expression of transcription factors……63

Figure 10. Maturation markers and CXCR3 in the group 1 ILCs of the CNS……64

Figure 11. Phenotypic profile of group 1 ILCs of the CNS……..65

Figure 12. Transcription factor and surface marker expression during EAE……66

Figure 13. Cytokine production of group 1 ILCs of the CNS……...67

Figure 14. Distribution of group 1 ILCs within the CNS compartments……69

Figure 15. Proliferation of group 1 ILCs in the CNS during EAE……70

Discussion

Figure 16. Phenotype of CNS-ILC1s and NK cells……..82

3 Introduction

3.1 Overview of the immune system and its innate and adaptive arms

Biological organisms are continuously exposed to a myriad of potential pathogens. One of the key evolutionary assets of all organisms was the development of a mechanism by which those threats could be identified and eliminated. This mechanism is what we call the immune system, manifested in different ways in bacteria, plants, fungi, invertebrates and vertebrates.

In vertebrates, the immune system is classically divided into the innate and the adaptive arm. The innate immune system represents the first line of defense and is considered to be fast, unspecific and not memory generating. The innate immunity consists of anatomical barriers like the skin; a humoral component, like the complement system and coagulation cascade; and a cellular component. The main cellular components of the innate immunity are the macrophages, neutrophils, dendritic cells, natural killer cells and the recently discovered innate lymphoid cells. They possess pattern recognition receptors on their surface that recognize molecular patterns common in pathogens, injured and transformed cells. This receptor activation leads to a fast response that results in the secretion of inflammatory molecules including chemokines and cytokines that attract and regulate the function of other immune cells. In addition, the innate immune cells are able to directly eliminate pathogens by different mechanisms such as phagocytosis or cytotoxicity. Due to its early response, the activity of the innate immunity shapes the nature of the adaptive immune response.

The adaptive immune system, also known as acquired or specific immune system, is mainly comprised of T and B lymphocytes. Their key features are their specificity, conferred by a huge variety of antigen-specific receptors; and their capacity to generate immunological memory, which is the ability to mount a fast response after the exposure of a previous encountered antigen, for instance, after re-infection with the same pathogen. The humoral component of the adaptive immune system are the antibodies, produced by the B cells. Antibodies bind specifically to antigens contributing to pathogen clearance in different ways, for example by tagging them for recognition by phagocytes or activating the complement system. In contrast to the innate immune system, in which pattern recognition receptors are encoded in the germline, the antigen-specific receptors of the T and B lymphocytes are generated in the cell during the lifetime of the organism by a process of somatic recombination. In this way, a few hundred different gene segments are recombined in different ways to generate thousands of different receptors (1). When a T or B cell receptor binds to its antigen, a process of clonal expansion that lasts several days starts until the cell mature into an effector cell. Once the antigen is removed, some cells persist in the form of memory cells that will react

quicker to the next infection with the same antigen. The immune response has to be tightly regulated to avoid collateral damage and the development of autoimmunity. Therefore, various subsets of cells are specialized in curving the immune response. Among them, the regulatory T cells (Tregs) are the most notable immunosuppressive cells. Moreover, both innate and adaptive immune cells possess regulatory functions.

The immune cells are generated in the bone marrow from hematopoietic stem cells (HSC), where most of them also mature (1). T cells, in contrast, undergo a maturation process in the thymus. The process of hematopoiesis gives rise to all cellular components of the blood including the red blood cells. In this process, the HCS differentiate into a myeloid progenitor or a lymphoid progenitor. The common myeloid progenitor cells further differentiate to give rise to granulocytes (i.e neutrophils, basophils and eosinophils), monocytes (macrophages and dendritic cells) erythrocytes and mast cells. On the other hand, the common lymphoid progenitor cells differentiate into T and B lymphocytes, NK cells and innate lymphoid cells.

Mature immune cells travel through the lymphatic system and the blood stream and come together in the secondary lymphatic organs such as the spleen and lymph nodes. The lymph nodes are connected with an extended network of lymphatic vessels and capillaries that span the whole body and drain fluid from the tissues and blood. It was long believed that the central nervous system was the only structure that lack a lymphatic drainage. However, a meningeal lymphatic system that drains cerebrospinal fluid to the deep cervical lymph nodes was recently discovered (2). In the lymph nodes, innate and adaptive cells reside and act in concert in highly organized structures, encounter survival signals, and are activated by antigen presenting cells (APCs) to induce an immune response.

In addition to the classical lymphoid organs, it has been increasingly recognized that other organs like the lung and the liver contain immune cell niches and perform immune functions. For example, the liver is a repository of numerous innate and adaptive immune cells. Also, it has a key role in the detection and clearance of pathogens from blood, and has a major role in eliminating activated T cells (3,4). In the case of the lung, it was shown that it can serve as a site of memory and effector T cell reactivation (5). In addition, it is the organ with the highest frequency of NK cells among other lymphocytes (6).

In fact, all tissues possess resident immune cells that continuously scan the environment and participate in immune defense. The most recent type of tissue resident immune cell described are the innate lymphoid cells (ILCs). Intensive research in the biology of ILCs in the last decade has deepened our appreciation of the many roles of the immune system in functions as diverse as tissue morphogenesis, regeneration and metabolism and their crucial interactions with the microbiome and the nervous system to maintain tissue homeostasis (7).

3.2 Innate lymphoid cells, the counterpart of T lymphocytes

The establishment of the ILC family is very recent. The natural killer (NK) cells were the first members to be recognized in 1975 as cells that displayed spontaneous or “natural” cytotoxicity against tumor cells without previous immunization (8,9). The lymphoid-tissue-inducer (LTi) cells were then described in the late nineties as essential cells for the development of lymph nodes and Peyer’s patches (gut associated lymphoid tissue) (10). Subsequently, newly discovered unorthodox innate immune cells were given various names like the interleukin (IL)-22-producing mucosal NK-22 cells (11) or the IL-13-producing nuocytes (12). Finally, a proposal for a uniform nomenclature in 2013 (13) categorized these cells into three different groups that mirror the classification of T cells. Those are the group 1 ILCs that include the NK cells and ILC1s; the group 2 ILCs composed by the ILC2s; and the group 3 ILCs that include the LTis and the natural cytotoxicity receptor (NCR) positive and negative ILC3s. The latest update in the classification expand it into five subsets – NK cells, ILC1s, ILC2s, ILC3s and LTis – based on their developmental trajectories (7). The ILC1s, ILC2s, ILC3s and LTi are collectively referred as helper ILCs and are specially enriched in mucosal tissues. In the present thesis, I will refer collectively to NK cells and ILC1s as group 1 ILCs. The ILC classification applies for both human and mice. Although helper ILCs have been more extensively studied in mouse models, the presence of all ILC groups have been confirmed in humans multiple times (summarized in (14). However, some aspects of human ILC development does not seem to be the same as in mice and requires further refining (14).

ILCs, like the T and B cells, originate from the common lymphoid progenitor (CLP), but in contrast to their adaptive counterpart, they lack rearranged antigen-specific receptors. The CLP gives rise to the innate lymphoid progenitor (CILP) that differentiates into the NK cell precursor (NKP) or the common helper innate lymphoid precursor (CHILP). The CHILP in turn, differentiates into the LTi precursor (LTP) and the ILC precursor (ILCP) from which the ILC1, ILC2 and ILC3s develop. Each differentiation step requires the expression of a set of transcription factors depicted in Figure I. In contrast to NK cells, helper ILCs require the expression of the transcription factor GATA-3 for their development (Yagi 2014) and are phenotypically defined by the surface expression of IL-7 receptor alpha (IL-7Ra) or CD127. IL-7 is required for the maintenance of ILCs, and its deficiency (or IL-7Ra deficiency) largely reduce the number of ILC2s and ILC3s in the tissues (15). In turn, IL-15 is more important for ILC1 and NK cell maintenance (16–18).

The ILCs are considered as the innate counterpart of the T cells because their transcriptional programs and cytokine secretion mirror those of the different T cell subsets. The similarity in the effector functions of T cells and ILCs have raised the question of whether ILCs

have a “redundant” role in immunity. Indeed, the function of ILCs may overlap with their adaptive counterparts in many contexts. However, mounting research is showing that ILCs and T cells cooperate, relay and complement each other (19–22). Furthermore, the ILCs serve as an interface between the external environment and the adaptive immune system. Thus, the regulation of ILC functions is key in the maintenance of tissue homeostasis and the limitation of autoimmunity (23,24).

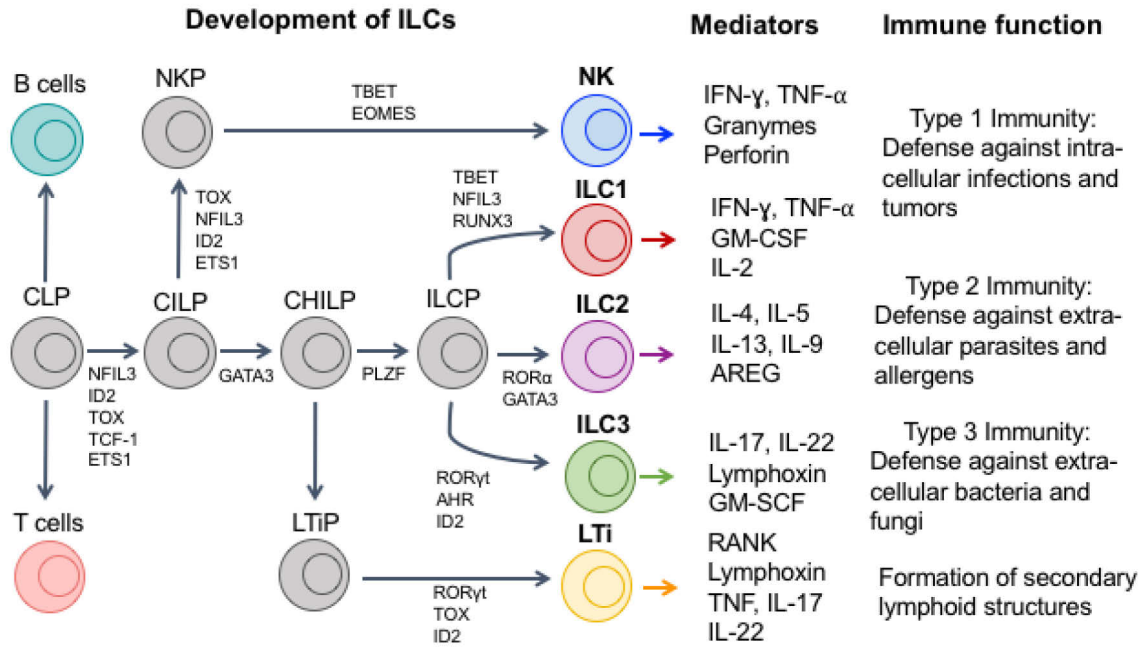

Figure I. Development and function of ILCs. Precursor cells are shown in grey. The transcription factors needed for their differentiation are indicated in the arrows. Each subset secretes different effector molecules upon activation that mediate important immune functions. Abbreviations: CILPs (common innate lymphoid pro- genitors), CLPs (common lymphoid progenitors), CHILPs (common helper innate lymphoid progenitors), LTiPs (lymphoid tissue inducer progenitors), ILCP (innate lymphoid cell precursors), NFIL3 (nuclear factor IL-3 induced), Id2 (inhibitor of DNA binding 2), TOX (thymocyte selection associated high mobility group box protein), TCF-1 (T cell factor 1), ETS1 (avian erythroblastosis virus E26 homolog-1), GATA3 (GATA binding protein 3), PLZF (promyelocytic leukemia zinc finger), T-bet (T-box transcription factor), Eomes (Eomesodermin), RUNX3 (runt-related transcription factor 3), RORa (RAR-related orphan receptor a), Bcl11b (B cell lymphoma/leukemia 11B), Gfi1 (growth factor independent 1), RORγt (RAR- related orphan receptor γt), and AhR (Aryl hydro-carbon receptor). Figure based on schemas from (7).

Next, an overview of each subset is presented, with special emphasis on the group 1 ILCs (i.e. NK cells and ILC1s).

3.2.1 NK cells

NK cells constitute the cytotoxic member of the ILC family and is thus considered to be the counterpart of the cytotoxic CD8+ T cells. Like the CD8+ T cells, NK cell development depends on the T-box transcription factor (T-bet) and Eomesodermin (Eomes) (25–27) and secrete the signature type I cytokine interferon gamma (IFN-γ). Also, IL-15 is essential for the development, homeostasis and function of NK cells (28,29). In addition, IL-12 and IL-18 play important roles in NK cell maturation and activation (30).

NK cells are crucial in protecting the host against intracellular infections and cancer. They possess a vast array of inhibitory and activating receptors with which they recognize healthy from transformed cells. The balance between inhibitory and activating signals and the cytokine microenvironment determine the nature and intensity of the NK cell response. Healthy cells constitutively express major histocompatibility complex class I (MHC-I) receptors on their surface. NK cells are “educated” to tolerate these MHC-I-expressing self-cells by detecting them with their inhibitory receptors, such as the killer cell immunoglobulin-like receptors (KIRs) in humans, the lectin-like Ly49 dimers in the mouse and the lectin-like CD94-NKG2A heterodimers in both species (31). When they engage to MHC-I molecules, cell lysis is inhibited, but when stressed, tumoral or infected cells downregulate MHC-I membrane expression, the lack of inhibition activates NK cell cytotoxicity (32). In turn, cells in distress express ligands that can be recognized by activating receptors triggering NK cell cytotoxicity. Many of these receptors are conserved in mice and humans. In fact, NKp46 (natural cytotoxicity triggering receptor 1) serves as a prototypical marker of NK cells in various species ranging from mice to monkeys and humans (33). Cells that have been opsonized with antibodies can also be recognized by NK cells and elicit a cytotoxic response by interacting with the Fc-receptor CD16 in a process called antibody-dependent cellular cytotoxicity (ADCC) (34). NK cells exert their killing abilities by releasing granules that contain perforin and granzymes. Perforin is a pore-forming protein and granzymes are proteases that together induce target cell apoptosis by activating caspases and damaging mitochondria and DNA (35). In addition, NK cells upregulate the apoptosis inducing ligands FasL and TRAIL upon activation (36). Besides being effective killing machines, NK cells produce cytokines and influence the function of other immune system cells. NK cells produce immunoregulatory cytokines including IFN-ɣ, tumour necrosis factor-alpha (TNF-α), IL-10 and granulocyte-macrophage colony stimulating factor (GM-CSF). They stimulate other innate immune system cells such as neutrophils and macrophages and shape the T cell response. Monocyte and dendritic cell derived cytokines IL-12, IL-15 and IL-18 as well as the T cell derived IL-2 modulate NK cell proliferation, maturation and function (31,37–39).

In the last years, it has emerged that NK cells display adaptive features in both human and mice. In particular, it has been shown that a subset of NK cells that recognizes cytomegalovirus (CMV) infected cells via the Ly49H receptor in mice and the NKG2C receptor in humans, display memory features. This subset of NK cells expands upon activation, contracts and differentiates into a long-lived memory subset that persist several months and is able to mount a recall response to a CMV reinfection, protecting the host better than naïve NK cells (40–42).

Different maturation and functional NK cell subsets have been described in both humans and mice. In humans, NK cells are phenotypically defined by the expression of CD56 and CD16. The $CD56^{dim}$CD16+ NK cells display a high cytotoxic activity, while the $CD56^{bright}$CD16- NK cells have a lower cytotoxic potential but proliferate more readily upon stimulation and secrete higher amounts of cytokines (43). The $CD56^{bright}$ subset is considered the immature subset as they can differentiate into $CD56^{dim}$ cells (44,45).

Mouse NK cells lack CD56 expression and are phenotypically defined by the expression of the natural cytotoxicity receptors NKp46 and NK1.1 depending on the mouse strain. The mouse strain used in the present study, C57BL/6, expresses both the NKp46 and NK1.1. The integrin CD49b (also known as DX5 or a2b1 integrin) is also constitutively expressed in NK cells and its expression was shown to be dependent on Eomes (26), which also regulates the expression of other NK cell-specific genes like Gzmb (Granzyme B) and Prf17 (Perforin) (46). Therefore, surface markers associated with Eomes expression, such as CD49b, represent reliable markers to distinguish NK cells from other ILC subsets.

The maturation subsets of NK cells can be distinguished by the expression of CD27 and CD11b. First, NK cells acquire the expression of CD27 and as the cell matures CD11b is expressed and CD27 is lost. Hence, the sequential developmental stages are divided as follows: CD27-CD11b-, CD27+CD11b, CD27+CD11b+ and CD27-CD11b+ (47). For simplicity, the immature NK cells can be designated as CD11b- and the mature as CD11b+. Similar to the human, immature CD11b- NK cells are more proliferative, secrete more cytokines and possess lower cytolitic granules than the mature CD11b+ NK cells (47,48).

DNAM-1 (DNAX accessory molecule, CD226) is another marker proposed to identify the maturation status of NK cells. DNAM-1 is an adhesion and costimulatory molecule that binds to the poliovirus receptor ligands nectin-2 (CD112) and PVR (CD155) (49). It plays important roles in mediating NK cell functions, like cytotoxicity against tumors, secretion of IFN-γ and generation of memory NK cells (50–53). It was shown that DNAM-1+ NK cells could be considered immature since they can differentiate into DNAM-1- NK cells. The DNAM-1+ cells are more proliferative, cytotoxic and secrete more cytokines than the DNAM-1- NK cells, which secrete more chemokines and were assigned a sentinel function (54).

3.2.2 ILC1s

ILC1s and NK cells share many similitudes. In fact, ILCs have been mistaken by NK cell for many years, for example, the liver ILC1s were initially thought to represent immature NK cells (55). Like NK cells, ILC1s express NKp46 and NK1.1, secrete IFN-γ and depend on T-bet and IL-15 for their development. However, ILC1s do not need Eomes for their development, possess low cytolitc granules and thus are considered non-cytotoxic. ILCs are deemed to contribute to the immune response by secreting higher levels of cytokines than NK cells including TNF-α, GM-CSF and IL-2 (7,56,57). However, ILC1s usually express the apoptosis inducing ligand TRAIL and accordingly, it has been shown that ILC1s can mediate TRAIL-dependent tumor killing in the liver (58).

Eomes expression is important to repress the integrin CD49a (alpha 1) and induce CD49b, therefore, ILC1s express only CD49a, which is used as a marker to distinguish them from NK cells (17,27). ILC1s express other ILC-specific markers like CD90 and CD127 (59). However, the expression of CD127 in ILC1s is more heterogeneous than initially assumed. For example, the gut lamina propia ILC1 are CD127+ while the gut intraepithelial ILC1s are CD127- (60). In the liver, only a fraction of the ILC1s express CD127 (61). Recently, the inhibitory CD200R1 was proposed to be a useful marker to discriminate ILC1s, as it is not expressed in NK cells (62). Furthermore, ILC1s express the transcription factor Hobit (also known as Zfp683), which has been implicated in controlling lymphocyte tissue-residency and survival (63). Hobit-deficient mice exhibit a specific reduction in liver ILC1s as thus has served as a model of ILC1 deficiency (62), although a more comprehensive analysis of the effect of Hobit deficiency in other organs is still needed..

ILC1s are essential for clearance of viral infections and other intracellular and extracellular pathogens like the parasitic protozoan *Toxoplasma gondii* and the bacteria *Clostridium difficile*. In the case of viral infections, it was demonstrated with a mouse model of CMV infection that ILC1s provide the earliest source of IFN-γ to the infected tissues including the sites of entry like the lung, skin and mouth and the sites of infection like the liver and spleen. This was crucial to restrict virus replication and dissemination and improved host survival. Interestingly, the group also showed ILC1s are more responsive to IL-12 than NK and NKT cells in the liver (62). In *T gondii* infection, IFN-γ contribute to the recruitment of inflammatory monocytes (Schulthess 2012) and induce reactive oxygen species (ROS) in infected cells, restricting parasite replication and growth (64). ILC1s produce IFN-γ and TNF-α and limit T. *gondii* replication in the intestine (17,60,65). Host defense against *C. difficile* acute infection also requires ILC1-derived IFN-γ (66). On the other hand, ILC1-derived IFN-γ can also have detrimental effects in some contexts. For example, it was shown that in the adipose tissue, the

ILC1s proliferate and accumulate during high fat diet, producing IFN-γ and contributing to the polarization of M1 macrophages, which are associated with obesity-induced insulin resistance (67).

In the context of tumor microenvironment using mouse models of primary and transplantable tumors, it was shown that ILC1s were unable to restrain tumor growth and metastasis, in contrast to NK cells, which played a critical protective role (68). However, a subset of “cytolitic ILC1s” have been described in various organs that are able to mount strong cytotoxic responses against tumors. Contrary to conventional NK cells and helper ILC1s, the cytolitic ILC1s do not depend on the transcription factor Nfil3 for their development (69–71) and their ontogeny is yet to be elucidated.

3.2.3 ILC2s

ILC2s are the counterpart of Th2 cells. As such, they mount type 2 immune responses that are specialized in protecting the host against large allergens and parasitic worms. ILC2s depend on GATA-3 and retinoic acid receptor-related orphan receptor-α (ROR-α) for their development and function. The cytokine IL-7 is also necessary for their development. ILC2s produce the cytokines IL-4, IL-5, IL-9 and IL-13 and respond to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). They are enriched in barrier surfaces such as the skin, lung and intestine and can be recognized by the expression of CD90, CD127 and the IL-33 receptor subunit T1-ST2 (7). ILC2s are essential in the coordination of the immune response against parasitic worms such as *Nippostrongulus brasiliensis*. The cytokines they secrete regulate monocyte and granulocyte responses and induce smooth muscle contractility that promote parasite expulsion (72,73). Furthermore, ILC2s are associated with tissue repair by producing the ligands for the epithelial growth factor receptor such as amphiregulin, which is required for epithelial cell differentiation and proliferation, as shown in a model of intestinal inflammation (74) and influenza-induced tissue damage in the lung (75). Interestingly, ILC2s are constitutively present in visceral adipose tissue where they can regulate adiposity and insulin resistance (76).

3.2.4 ILC3s

ILC3s are the counterpart of Th17 cells, which are involved in the immune response to extracellular bacteria. They depend on the transcription factor retinoic acid receptor-related

orphan receptor-γt (RORγt) for their development and secrete the cytokines IL-17 and IL-22. ILC3s are activated by IL-23 and IL-1β and predominantly reside in mucosal tissues. ILC3s express the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that acts as an environmental sensor, as it is activated by various compounds including toxins, tryptophan metabolites, dietary products and bacterial pigments (73). AHR activation controls the survival of ILC3s (77), and is essential in maintaining gut homeostasis. For example, the activation of AHR by tryptophan metabolites from commensal bacteria induce the secretion of IL-22 and antimicrobial peptides that prevents bacterial dissemination and inappropriate immune responses against them (78,79). In addition, ILC3s can promote a tolerogenic state in the gut by regulating T cells responses against commensal bacteria (21). IL-22-producing ILC3s are also critical for defense against bacterial infections such as *Citrobacter rodentium,* which is the mouse equivalent of human *Escherichia coli* infection that causes acute colitis (80,81). This cytokine also promotes tissue repair and regeneration in the inflamed intestine (Sawa 2011) and limits tissue damage in allergic lung disease (82).

ILC3s are CD127+ and can be divided in NCR+ and NCR- ILC3s. NCRs include NKp46 and NKp30 in humans and mice and NKp44 in mice. NKp46+ ILC3s were first discovered and described as atypical NK cells derived from the LTis (83,84). The distinction in the function between these two subsets is not clear, however, NKp46+ ILC3s have been shown to have anti-tumor properties (85–87).

3.2.5 LTi cells

Lymphoid tissue inducer cells depend, like the ILC3s, on the transcription factor RORγt but arise from a different progenitor than the rest of the helper ILCs. LTi cells are CD127+ and depend on IL-7 for their survival and differentiation (88). LTi cells are NCR-, express the chemokine receptor CCR6 and a subset is CD4+. LTi cells are present early in fetal development and coordinate the formation of lymph nodes and Peyers patches (10,89). LTi cells produce lymphotoxins that induce the secretion of chemokines and adhesion molecules in stroma organizer cells, which attract lymphocytes during fetal development to form lymphoid structures (90). After birth, LTi-derived lymphotoxins regulate gut immunity (22).

3.2.6 ILC plasticity and heterogeneity

One of the features that have raised interest in the study of ILC subsets is their plasticity, that is, the ability of one ILC subset to differentiate into a different ILC subset. ILCs exhibit tissue specific characteristics and dynamically respond to the stimuli present in the tissue microenvironment by regulating their phenotype and function (87). Furthermore, a great heterogeneity can be found within an individual ILC subset, as it was revealed in single cell transcriptome analysis of the ILCs present in the small intestine lamina propria (91). In that study, the authors found four ILC1, four ILC2 and five ILC3 functional subsets based on their transcriptional profiles. Interestingly, some of these sub-subsets were altered after microbiome manipulation, corroborating the dependency of ILC phenotype and functionality on tissue microenvironment signals.

TGF-β can drive the differentiation of NK cells into ILC1s. TGF-β signaling repress Eomes and induces the expression of ILC1 markers such as CD49a and TRAIL (92). Interestingly, in the TGF-β-rich tumor microenvironment, an intermediate subset that express both CD49a and CD49b was found, termed intermediate-ILC1s (intILC1s). This subset displays features of both NK cells and ILC1s and exhibits a higher proliferation rate (68). It is therefore possible that the conversion of NK cells into ILC1s after TGF-β stimulation goes through an intILC1 stage.

Another notable example of ILC plasticity is the ability of ILC3s to convert into ILC1s. After IL-12 stimulation, ILC3s downregulate RORγt and upregulate Tbet and NCR expression acquiring the ability to secrete IFN-γ (65,93,94). The ILC1s derived from an ILC3 lineage have also been called “ex-ILC3s. ILC1s can in turn differentiate into ILC3s after IL-23, IL-1b and retinoid acid (RA) stimulation (11,95). This conversion of “homeostatic” IL-22-producing ILC3s into “inflammatory” IFN-γ-producing ILC1s was shown to be important to fight *Salmonella* infection in the gut. However, ILC1s also promoted enterocolitis (94). In line with this, an accumulation of ILC1s was found in the inflamed intestine of Crohn's disease patients (65).

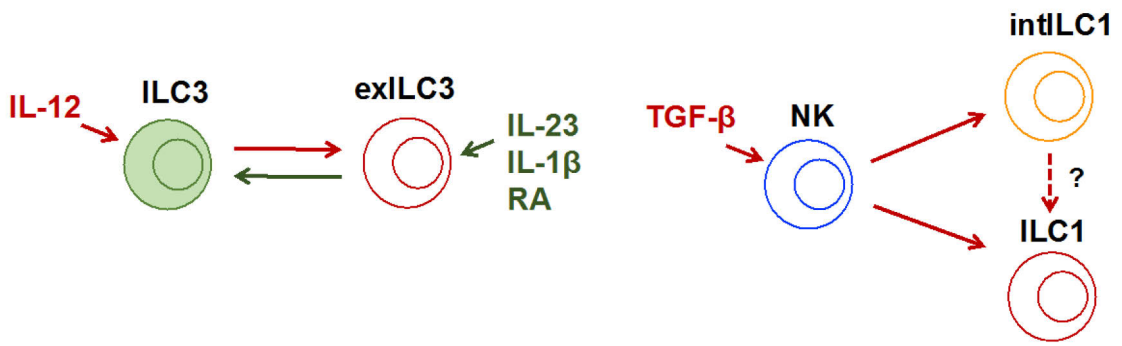

Figure II. Plasticity of NK cells, ILC1s and ILC3s. Stimulation with IL-12 can induce ILC3s to adopt an ILC1 phenotype (exILC3) with a corresponding downregulation of RORγt and upregulation of T-bet. In turn, differentiation of ILC1s into ILC3 can be induced by IL-23 stimulation and accelerated by IL-1b and RA. NK cells can adopt an intILC1 or ILC1 phenotype in response to TGF-β stimulation. It remains unclear whether intILC1s differentiate into ILC1s.

3.3 Tissue residency, migration and circulation of ILCs

Helper ILCs are tissue resident cells, while NK cells continuously circulate in the blood stream and lymphatic system. In humans, NK cells comprise around 15% of the total lymphocyte population that circulate in the blood, though only a minute fraction of helper ILCs can be found in the blood of healthy subjects (Brunotte-Strecker and Infante-Duarte unpublished data). NK cells are enriched in the red pulp of the spleen and are able to migrate to lymph nodes and sites of infection. Most of the initial studies on NK cells were performed using NK cells derived from mouse spleen. Splenic NK cells are therefore the prototypical NK cells, which are also called conventional NK (cNK) cells. Subsequently, tissue-resident NK cells were described in thymus, liver, uterus and salivary glands (96–99). It was later recognized that most of this tissue-resident NK cells represent ILC1s (57,100). However, some organs maintain a great diversity of particular NK cell subsets that are difficult to classify within the ILC spectrum, like the uterine NK cells. Thus, uterine tissue harbors Eomes−CD49a+ ILC1s, Eomes+CD49a− cNK cells and Eomes+CD49a+ tissue resident NK cells (99). Each subset seems to have a specialized function during reproductive life. Tissue resident NK cells expand during early gestation while cNK cells expand during late pregnancy. Interestingly, ILC1s expand during second pregnancies suggesting a potential memory role of this subset (101).

Furthermore, properties that many tissue-resident NK cells have in common and that makes them hard to classify into the ILC1 subset is their cytotoxicity potential (61,96) and their development independent of the transcription factor Nfil3 (96,98,102,103). Therefore, these cells have also been called cytolitic or killer ILC1s (70,71). The dependency on Nfil3 was tested using the mouse deficient in this transcription factor. However, it has been argued that the impact of this transcription factor in the Nfil3-deficient mouse is affected by the environment (like ongoing inflammation or variable housing conditions). Thus the dependence on Nfil3 is most likely insufficient to define a cell lineage, especially in endocrine organs where the tissue microenvironment is enriched with factors not present in other tissues (57,104).

Tissue residency of ILCs has been demonstrated in parabiosis experiments. In the parabiotic mouse model, the skin of two mice are stitched together in the flanks; the wound healing process join the bloodstream of the animals through capillary networks, which results

in the two mice sharing a common bloodstream. By joining mice that express different alleles of CD45 (CD45.1 vs CD45.2), the presence of cells originating from one mice can be detected in the tissue of the conjoined mice. In this way, it was shown that NK cells recirculate, as their composition in diverse organs of the parabiont was chimeric (that is, contained both CD45.1+ and CD45.2+ NK cells). On the other hand, ILC composition displayed no chimerism as more than 95% of them expressed the host CD45 allele, thus demonstrating that ILCs do not recirculate. (105). An exception to this is the ILC2s, it was recently shown that upon helminth infection, inflammatory ILC2s are able to circulate and migrate to diverse organs (106).

3.3.1 Chemokines and adhesion molecules

Chemokines are a large family of small proteins that mediate chemotaxis of nearby cells. Their receptors are composed by a superfamily of G-protein-coupled seven-transmembrane receptors named according to the number of aminoacids between their conserved cysteine residues; namely the C, CC, CXC and CX3C families. About 50 chemokines and 20 functional receptors are known. A given receptor usually interacts with more than one chemokine and several chemokines can bind to more than one receptor (107), creating a very complex network in which redundancy may exist. However, detailed investigations have revealed that many axis in this network are highly fined-tuned. For example, the different ligands of CXCR3 – CXCL9, CXCL10 and CXCL11 – bind to their receptor with different affinities eliciting differential cell signalling responses (108).

Cell adhesion molecules (CAMs) include many families of transmembrane receptors that interact with the cytoskeleton in their intracellular domain and with components of the extracellular matrix (ECM) or other adhesion molecules in its extracellular domain. Among the many CAMs that mediate lymphocyte migration and retention are the integrins. Integrins are heterodimers composed by an alpha chain and a beta chain. Mammals have 24 alpha subunits and 9 beta subunits (109). As mentioned above, ILC1s express the apha-1 integrin CD49a, this subunit dimerizes with beta-1 integrin subunit to form a complex that binds to collagen and laminin present in the ECM of the tissues (110). Intraepithelial ILC1s express the integrin alpha-E CD103, this subunit forms a complex with beta-7 integrin that is able to bind to E-cadherin found in epithelial cells (111).

An interplay between the expression of chemokine receptors and adhesion molecules determine the migratory potential of a lymphocyte. The expression of such receptors and molecules is highly dynamic in the lymphocyte lifespan and is determined by developmental and environmental cues, such as inflammation. During steady state, tissues constitutively express chemokine ligands that act as “homing” signals to attract lymphocytes, like the ligands

of CCR7 – CCL19 and CCL21 – that are abundant in secondary lymphoid organs (112,113). During inflammation, a different set of chemokine ligands might be upregulated to attract a diverse range of immune cells. Specialized cell subsets can be recognized by their chemokine receptor expression. In this line, we showed that CX3CR1 is expressed in mature NK cells in both humans and mice, and that it mediates the migration of mature NK cells into the inflamed CNS (114,115). In the case of ILC1s, the expression of CXCR6 and sometimes CXCR3 have been described as a distinctive feature of this ILC subset. CXCR6 is associated with homing to the liver as its ligand, CXCL16, is constitutively expressed in the liver sinusoidal endothelium (116). CXCR6 has been associated with the homeostasis and function of liver memory ILC1s/NK cells (117). CXCR3 ligands are induced in the tissues during inflammation by IFN-γ (118,119). This mechanism is crucial for the mobilization of activated and memory T cells to sites of inflammation (120,121). The role of this cytokine in ILC1 and NK cell recruitment has been poorly studied.

Signalling through chemokine receptors can modulate other functions than chemotaxis and activate differential signalling cascades depending on the ligand. This was shown in murine CD4+CXCR3+ T cells, in which CXCR3 signalling induced by the ligands CXCL9/10 promoted a polarization to TH1/17 cells, while the activation with CXCL11 promoted a Th2/Tr1 polarization (122). It is unclear whether this biased signalling is present in CXCR3-expressing group 1 ILCs.

Chemotactic molecules present in the bloodstream induce the egress of lymphocytes expressing their respective receptors from the tissues. In particular, a gradient of sphingosine-1-phosphate (S1P), a signalling sphingolipid that binds to its G protein-coupled receptors S1P1-S1P5, is present in the blood provoking the lymphocytes to egress tissues and enter the circulation. The lectin CD69 inhibits this process by forming a complex with S1P1 in the cells, provoking its internalization and destruction (123–125). CD69 is also used as an early activation marker in lymphocytes. In line with this, ILC1s constitutively express CD69, while NK cells upregulate this molecule only after activation.

3.3.2 Homing of immune cells into the healthy and inflamed CNS

The central nervous system is considered to be an immune privileged organ. This term refers to an organ that has a limited capacity to mount an immune response in the presence of an antigen. This concept was demonstrated in the middle of the 20th century with tissue grafts experiments. Tissue grafts are normally recognized as foreign and rejected by the immune system. A tissue graft implanted into the brain parenchyma is tolerated and able to

survive for a long time without rejection (126,127). The lack of a lymphatic system in the brain parenchyma and the particularities of the blood brain barrier (BBB) led to the hypothesis that the immune system had no access to the CNS parenchyma. In contrast to the blood vessels in the peripheral organs, the endothelial cells that form the blood vessels in the CNS are joined by tight junctions, rendering them almost impermeable to cells and solutes. The endothelial cells of the BBB are surrounded by pericytes, a basal membrane and astrocytic end-feet, which serve as support and provision of regulatory factors (128). The brain parenchyma is surrounded by cerebrospinal fluid (CSF) and three layers of meninges: dura mater, arachnoid mater and pia mater. The pia mater is a one-layer permeable membrane that is directly coating the brain parenchyma and associated blood vessels. Beneath the pia, the *glia limitants*, composed by basal membrane and astrocytic end-feet, represent another border to the brain parenchyma. Between the pia and the arachnoides, a subarachnoid space is formed where CSF flows. The arachnoid mater is adjacent to the dura mater. The dura mater is a fibrous membrane that contains arteries, veins and lymphatics, all of which are fenestrated and allow the transport of cells and solutes. The arachnoid matter is impermeable thanks to its tight junctions, representing the real CSF-blood barrier that separates the CSF bathed brain parenchyma from the contents of the dural lymphatics and vessels (129). The CSF is constantly produced in the choroid plexus (CP) in the brain ventricles. The choroid plexus consists of a layer of epithelial cells surrounding a core of capillaries and connective tissue, also called stroma. The endothelial cells of the capillaries of the CP are fenestrated allowing the entry of immune cells into the CSF. However, the epithelial cells of the CP contain tight junctions forming a barrier between the CSF and the blood (blood-CSF barrier). This complex architecture makes the CNS a very difficult place to access. However, it is currently accepted that immune surveillance – a continuous process whereby the peripheral immune system is able to monitor an organ for signs of infection or tissue damage – occurs in the CNS and that it is essential to maintain CNS homeostasis (130–132). Indeed, one of the early experiments on graft rejection showed that a condition to elicit graft rejection in the brain was to first expose the animals to the graft antigen in the periphery (126). This indicates that the T cells that are activated in the periphery by a CNS antigen are able to reach the brain and mount an immune response. This is precisely the principle of the animal model for autoimmune inflammation, the experimental autoimmune encephalomyelitis (EAE), which will be further described in the following section. This model has been instrumental for the understanding of the recruitment of immune cells to the CNS.

The recently rediscovered dural lymphatics were shown to have a connection with the deep cervical lymph nodes, which enables the passage of cells and macromolecules from the CNS into the periphery (2). In addition, drainage of antigens present in the CSF can reach the nasal and deep cervical lymph nodes by leaking through the cribriform plate into the nasal

mucosa (133,134). CSF can also exit through the arachnoid villi and reach the venous system in dural venous sinuses (135). Immune cells in turn, can enter the CNS through the choroid plexus, leptomeninges (arachnoid plus pia matter) and the perivascular spaces. However, the breaching of the above mentioned barriers that protect the brain parenchyma needs the inflammation-induced release of several factors including ECM remodelling proteases such as matrix metalloproteinases (MMPs), cytokines, chemokines and adhesion molecules (136,137). Chemokines play important roles in mediating the localization and barrier crossing of immune cells into the CNS. For example, chemokines like CXCL12 and the CXCR3 ligands are expressed in perivascular spaces and limit the localization of immune infiltrates into this space limiting inflammation in EAE (138,139). We showed that the expression of CX3CR1 in mature NK cells is needed for the infiltration of this protective subset into the inflamed brain (115).

Under steady state, immune cells are found in CNS compartments. The CP stroma, the subarachnoid space and the lining of the brain ventricles contain dendritic cells and macrophages that are able to present antigen to T cells (140–142). Interestingly, permeability of the CP epithelial layer was shown to be regulated by IFN-γ derived from resident effector memory T cells (defined as CD4+CD44^{high}CD62L^{low}) (143,144). Importantly, the possible role of IFN-γ-secreting group 1 ILCs in regulating CP permeability has not been explored.

In humans, the CSF of individuals with non-inflammatory diseases is found to contain central memory T cells (defined as CD4+CD45RA-CD27+CD69+) (145) and immature CD56^{bright} NK cells (146). The brain parenchyma is enriched with its own immune system cells, the microglia. Microglia colonize the CNS in early stages of embryonic development from primitive yolk sac macrophages (147). They serve varied functions during CNS development and regulate homeostasis in the adult brain (148). Microglia can be distinguished from the peripheral derived immune cells by a lower density of CD45 expression and a high expression of CD11b and CX3CR1 (149).

In addition to microglia, other immune cells are detected in the healthy CNS. In our lab, we systematically detect CD3+, CD11b+ and NK1.1+ cells in thoroughly perfused CNS of healthy murine from diverse strains. Other groups have reported the presence of NK1.1+NKp46+ or NK1.1+CD49b+ cells in the healthy CNS (150,151). However, a detailed investigation of the composition of tissue resident ILCs within the CNS compartments is completely lacking. Given the obligate tissue resident nature of ILC1s and its role in tissue homeostasis and early response to inflammation demonstrated in other organs, it is of great interest to characterize the group 1 ILCs that reside in the CNS.

Next, multiple sclerosis, the major autoimmune disorder that involves a dysregulation in the homeostasis of CNS immunity will be introduced. Followed by the description of its animal model, EAE, and the implications of group 1 ILCs in MS and EAE etiology.

3.4 Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by demyelination and subsequent axonal degeneration. MS is the leading cause of long-term disability in young adults (152). In Germany alone, nearly 200 000 people have been diagnosed with MS (153). MS is three times more common in women than in men and affects more people that live above 40° latitude and those from Caucasian origin (152). These incidence data suggest a complex interplay between genes, hormones and environment that is still subject of thorough investigations. Demyelinating lesions in MS appear in the brain, optic nerve and spinal cord, causing a broad spectrum of symptoms that depend on the location of the lesion. The most common symptoms are visual, motor, sensory and autonomic deficits, but speech, cognitive and emotional functions can also be affected (154).

MS most frequently follows a relapsing-remitting (RRMS) course which is characterized by defined “attacks” of worsening neurological functions followed by “remissions” that involve partial or complete recovery. A lower number of patients are diagnosed with primary-progressive MS (PPMS) where a slow progressive worsening of neurological function occurs with no distinct relapses or remissions. In addition, about 65% of patients that suffer from RRMS develop a secondary-progressive (SPMS) course after 10-25 years of the disease (154).

The etiology of MS is not completely understood. Genetic factors that increase the susceptibility for MS are associated with some allelic variants of the human leukocyte antigen (HLA) system (the human MHC proteins), particularly HLA DR15 (155). In addition, genome wide studies have associated single-nucleotide polymorphisms (SNPs) of important immune regulators like IL-12RA and IL-7RA as heritable risk factors for MS (156,157). However, the probability that both identical twins are affected by the disease is only 25% (158–160) indicating that environmental factors play a significant role in initiating the disease. A protective role of vitamin D has been proposed since MS patients often present vitamin D deficiency and incidence data worldwide correlate with regions of low UVB ray exposure, which is essential for the vitamin D conversion in the skin. Vitamin D is an important immune modulator, and the supplementation of MS patients may have potential therapeutic effects (161).

Infections are possible triggers of MS. Epstein-Barr virus (EBV) infection in young adults increases the susceptibility to develop MS (162). While the reason of this correlation is still unclear, the mechanism probably involves EBV infected memory B cells (163,164). In addition, relapses are associated with common viral infections (165). The proposed mechanism by which an infection can trigger MS involves a process called “molecular mimicry”. This process occurs when structural motifs of a virus resemble molecules of the host, thus, the immune system attacks self-structures that are confused with foreign antigens. Another mechanism is the “bystander activation”, where the inflammatory response provoked by the infection causes the activation of pre-existent autoreactive T cells or reveals hidden autoantigens (also called epitope or antigen spreading) (166).

In the last decade, the role of the gut microbiome as important regulator of health has emerged. In line with this, investigations of gut microbiome of MS patients have shown that MS patients exhibit an altered gut microbial composition (known as dysbiosis) compared with healthy controls. These studies suggest that certain bacterial compositions have an anti-inflammatory role that contribute to limit autoimmunity (167).

Regardless of the factor that may trigger the disease, MS is considered to be T cell mediated. Autoreactive CD4+ T cells, activated by a CNS antigen (which still remains unknown) by local or peripheral APCs, migrate through the (perhaps already disrupted) BBB into the brain. There, they release cytokines, initiating an inflammatory response that activates local microglia and recruit other immune cells such as cytotoxic CD8+ T cells, B cells and macrophages. Although remyelination occurs, and it is associated with remissions, defective immune regulation as well as extended oligodendrocyte damage makes a complete remyelination a difficult task. As a consequence, the exposed axons degenerate leading to neuronal loss and permanent disability. Both Th1 (IFN-γ, TNF-α producing) and Th17 (IL-17 producing) responses, and a low suppressive activity of regulatory T cells (Tregs) have been associated with the disease (168). In addition, a central role of autoimmune B cells is also emerging in the field (169,170).

Magnetic resonance imaging (MRI) is the most sensitive tool to diagnose MS. With the use of a contrast agent containing the metal Gadolinium, white matter lesions in the brain and spinal cord can be identified. In addition to MRI, laboratory methods such as the analysis of inflammatory markers in the CSF are useful to complete the diagnosis (171). The first acute episode involving one or more focal demyelination sites is called clinically isolated syndrome (CIS), about 60% of CIS patients develop MS over the next 20 years (172). The expanded disability status scale (EDSS) proposed by Kurtzke in 1983 is currently used to evaluate the degree of neurological impairment in MS (173). The scale goes from 0 to 10, where 0 is no disability and 10 is death due to MS. The patient is scored after neurological testing of different

functional systems. MS patients with scores 1 to 4.5 are fully ambulant while the patients with score 5 to 9.5 have increasing degrees of disabilities that impairs daily activities.

Although MS has no cure, great advance has been made in the last decades in the development of disease modifying therapies that can substantially reduce the impact of MS on the lives of many patients. Many of those have been developed thanks to research with the EAE animal model of the disease, which has been instrumental to understand the disease mechanisms of neuroinflammation and the importance of immune cells in regulating CNS homeostasis.

3.4.1 Experimental autoimmune encephalomyelitis as the mouse model of MS

Not a single animal model can capture the heterogeneity and spectrum of MS. However, several animal models have been established that recapitulate certain aspects of human MS. In particular, the experimental autoimmune encephalomyelitis model has brought important insights into the pathogenic mechanisms of MS and contributed in the development and testing of novel therapeutic strategies.

EAE is induced by immunizing laboratory animals with myelin antigens and potent coadjuvants, which then develop an inflammatory disease of the CNS with various degrees of demyelination that correlate with clinical symptoms characterized by ascending paralysis. It was already noticed at the beginning of the 20th century that the anti-rabies vaccination could produce neuroparalitic complications in some subjects (174). The attenuated virus used in this vaccine was isolated from neural tissue cultures. Presumably, contamination of nervous tissue in the vaccine could have led to an autoimmune attack to the CNS manifested as an acute disseminated encephalomyelitis (175). Based on these observations, the first attempts to produce an experimental autoimmune encephalomyelitis were done 1933 in monkeys, using intramuscular injections of rabbit brain extract emulsions (176). The protocol was subsequently refined and applied to many other species including rabbits, guinea pigs, rats and mice. The mice model is currently the most popular model, and the availability of different transgenic and knock-out mice makes it a useful tool to investigate the role of a specific gene, protein or cell type in the disease pathogenesis.

An active immunization, or a passive adoptive cell transfer strategy can be used to induce EAE in mice. For the active strategy, depending on the mouse strain, different myelin peptides are used in the immunization protocol and different disease courses are achieved. When using the C57BL/6 mouse strain, a highly immunogenic (immunodominant) region of the myelin oligodendrocyte glycoprotein (MOG) is used to immunize the mice, usually the MOG_{35-}

$_{55}$ peptide. The peptide is prepared as an emulsion together with complete Freund's adjuvant that contains inactivated mycobacterium, which serves as a massive immune stimulant, and applied subcutaneously usually in flanks of the mouse. Injections of pertussis toxin are performed on the day of immunization and 48 hours after, which are necessary to augment the sensitization process. After 10 to 15 days, the mice develop a monophasic chronic EAE disease course, in which the animals may recover after a phase of clinical disease but do not present relapses. Similarly, the SJL mice are immunized with a proteolipid protein (PLP) antigen, usually $PLP_{139-151}$, to elicit an EAE with a relapsing remitting disease course. Although in the active immunization model, the disease is mainly mediated by autoreactive CD4+ T cells, the model has also allowed to discern the contribution of other immune cells to the pathogenesis of the disease, thanks largely to the genetic models available in the C57BL/6 background (177,178).

In the passive strategy, encephalytogenic T cells are obtained from the lymph nodes of an actively immunized mice. The cells are reactivated and expanded with the myelin peptide and APCs *in vitro* and then intravenously transferred into the recipient mice, usually a SJL mice that develops in a few days a relapsing remitting form of EAE. The passive EAE strategy has been useful to demonstrate the contribution of each CD4+ Th1 and Th17 cell subset in the development of the disease, to study the T-cell mediated immune surveillance and inflammatory tissue damage (175,179).

Overall, the EAE model is useful to study mechanisms of autoimmunity and neuroinflammation, but it does not recapitulate many aspects of MS. For example, inflammation and demyelination in active EAE is more common in the spinal cord, while in MS it is almost restricted to the brain. MS progression is difficult to study in EAE and the cell component is largely skewed to CD4+ T cells, while in MS, the contribution of other immune cells to disease pathology is also crucial (180). Therefore, toxic-induced models of demyelination such as the cuprizone model, which triggers apoptosis in oligodendrocytes and induces demyelination through mechanisms of oxidative injury, are more useful to study the process of de- and re-myelination (181).

3.4.2 The role of group 1 ILCs in MS and EAE

Early investigations of the implication of NK cell in MS revealed that MS patients have an impaired effector NK cell function. In specific, cytotoxicity of peripheral blood-derived NK cells against tumoral cells in *in vitro* assays was diminished in MS patients compared to healthy controls (182–184). Furthermore, longitudinal studies in RRMS patients showed that decline

in NK cell cytotoxic function preceded the appearance of new or enlarged of MRI lesions (185,186) and clinical relapses (187). Moreover, it was also found that the $CD56^{bright}$ NK cell subset presented a reduced capability to produce IFN-γ and to proliferate following cytokine activation (188). All these data suggested a protective role of NK cells in MS with a mechanism that is subset-specific. Indeed, our research group reported that $CD56^{dim}$ from MS patients had a reduced CX3CR1 expression, and that stable but not active MS patients had an elevated frequency of circulating $CD56^{bright}$ NK cells (189). In addition, we showed that the overall frequency of NK cells in CSF – which is enriched with $CD56^{bright}$ cells – is reduced in MS patients compared to patients of non-inflammatory neurological diseases (146). Interestingly, during pregnancy, MS activity is reduced while the proportion of circulating $CD56^{bright}$ NK cells augments (190). Thus, the $CD56^{bright}$ seems to have a protective immunomodulatory role in MS. In line with this, some therapies for MS like IFN-β (191) and daclizumab (192) results in an expansion of $CD56^{bright}$ NK cells that is accompanied by a reduction of disease activity. The mechanisms behind a protective role of NK cells in autoimmunity lie in the fact that NK cells can kill other immune cells, which becomes beneficial when those cells are autoreactive T cells. For example, it was shown that MS patients have a reduced expression of the co-activatory receptor DNAM-1 on both $CD56^{bright}$ and $CD56^{dim}$ NK cells. In addition, their antigen activated CD4+ T cells have a reduced expression of DNAM-1 ligand CD155. This provoked a decreased interaction of NK cells and T cells that resulted in a decreased NK cells cytolitic activity against the activated T cell. Interestingly, daclizumab treatment (an antibody that targets the IL-2R) restored that interaction by inducing an increase in CD155 expression on T cells and expanding NK cells (193). Similarly, it was shown that T cells from MS patients have an increased expression of HLA-E, which acts as a ligand of the inhibitory NK cell receptor NKG2A, resulting in a decreased ability of NK cells to suppress autologous CD4+T cell proliferation (194). Furthermore, MS treatments that employ monoclonal antibodies such as Alemtuzumab (which targets CD52 on T and B cells) and Rituximab (which targets CD20 on B cells) might work by inducing an antibody-dependent cell-mediated cytotoxicity by NK cells (195,196). However, NK cell cytotoxicity is a double-edged sword that could also have a tissue damaging role in the CNS during MS, as it has been shown in some EAE studies.

Investigations of the role of NK cells in the EAE model have at first sight drawn seemingly contradictory results. On one hand, detrimental roles of NK cells in EAE development were reported. IL-18 knockout mice, which have reduced NK cell numbers, are resistant to MOG-induced EAE. EAE could be established by treating the mice with IL-18, but this was not possible in NK cell depleted mice, suggesting a role of NK cells in the establishment of autoimmunity (197). In another study, the depletion of NK cells before MOG immunization with anti-NK1.1 or asialo GM1 treatment, decreased both the frequency and the extent of disease compared with EAE control mice (198). On the other hand, protective roles of NK cells have

been reported. For example, the same anti-NK1.1 depleting treatment was shown to induce a more severe EAE disease in both MOG-induced (199), and PLP-induced model (200). Moreover, the expansion of NK cells with anti-IL-2Ra antibody resulted in a less severe EAE in SJL mice together with a reduction of the activity of Th17 in the CNS (201). Like in MS, one of the mechanism proposed by which NK cells limit autoimmunity in the EAE model is by lysing autoreactive T cells. The interaction of the inhibitory receptor NKG2A with the ligand Qa-1 in T cells (mouse homolog of human HLA-E), has been shown as a mechanism by which T cells escape NK-mediated lysis. Thus, the blockage of this interaction with an antibody treatment resulted in an enhanced elimination of autoreactive T cells and decreased EAE disease course (202,203).

We and others have shown that the presence of NK cells in the CNS during neuroinflammation has a protective role. Mice deficient for the chemokine receptor CX3CR1 ($CX3CR1^{-/-}$) show a defective recruitment of NK cells into the CNS and an enhanced EAE severity (204). We showed using the same mice, that mature CD11b NK cells was the subset that failed to migrate to the CNS during neuroinflammation, and that this subset had a more potent response against autoreactive T cells (115). This is in line with another report that showed enhanced Th17 responses in the CNS of $CX3CR1^{-/-}$ mice (205). A detrimental role of NK cells present in the CNS was also reported, specifically in chronic stages of MOG-induced EAE (206). Liu and colleagues showed that NK cells accumulated in the subventricular zone (SVZ) in proximity to neural stem cells (NSC) during neuroinflammation. NSC sustained NK cells by secreting IL-15 and the presence of NK cells during chronic neuroinflammation resulted in the lysis of NSC limiting, thus, oligodendrogenesis and recovery. In sum, these data indicate that the role of NK cells in autoimmunity is largely dependent on the subset and disease stage investigated.

The role of ILC1s in MS has not been explored yet. The absence of ILC1s in the circulation and the low availability of tissue from patients limits the study of the role ILC1s in human disease. It is also important to note, that the above-mentioned investigations of NK cells in the EAE model have overseen the implication of ILC1s, either because ILCs were just recently formally described or due to the lack of tools to specifically target ILC1s. As both NK cells and ILC1s express NK1.1, many of the studies might have inadvertently targeted ILC1s; for example, when using anti-NK1.1 antibody treatments (198–200) or when visualizing NK1.1 cells that express a fluorescent reporter (206).

Few studies have addressed the role of ILCs in EAE. Using the EAE model induced by the adoptive transfer of Th17 cells, Kwong and colleagues showed that NKp46+ ILCs accumulate in the meninges and that there they promote a microenvironment that allows Th17 reactivation and parenchymal invasion. A specific role of ILC1s and NCR+ ILC3s was

suggested, as the specific deletion of Tbet in NKp46 expressing cells (using the $Tbx21^{f/f}$ NKp46-Cre^{+} mice) resulted in a less severe disease, while the use of a NK cell-specific deletion (using the $Eomes^{f/f}$ NKp46-Cre^{+} mice) did not affected the outcome of the disease (207).

In sum, NK cells and ILC1s have important and differential roles in modulating neuroinflammation. However, a thorough description of the phenotypic and functional characteristics of the group 1 ILCs that reside in the CNS is completely lacking. Therefore, the present doctoral thesis tackles this problem by providing a resource to allow the unambiguous identification of NK cells and ILC1s inside the murine CNS.

4 Aims of the Thesis

The identification of the mechanisms that mediate the trafficking of immune cells during homeostasis and inflammation has led to the development of successful treatments for autoimmune diseases such as MS. In this line, the trafficking of NK cells into the CNS deserves further investigation. In humans, multiple evidence suggest that NK cells have a beneficial, disease-limiting role in MS. In mice, the trafficking of mature CX3CR1+ NK cells into the CNS was shown to be beneficial in the EAE model of MS. However, immature NK cells are also present in the inflamed CNS. The chemokine receptors that mediate their migration to the CNS remain unclear. In addition, NK cells (defined as CD3-NK1.1+ cells) are already present in the CNS of healthy mice, which suggest a long-term tissue residency of these cells. Contrary to NK cells, helper ILCs reside in the tissues and do not recirculate. Taking into account the phenotypical similarities of NK cells and ILC1s, it is possible that some of the CD3-NK1.1+ cells present in the CNS constitute *bona fide* ILC1s.

Therefore, the overall aim of this thesis is to investigate into these two hypotheses:

1) The CNS immature NK cells migrate from the periphery using a specific chemokine receptor.

2) The CNS immature NK cells, or other subsets within the CD3-NK1.1- cells, constitute resident ILC1s.

The specific questions of these two hypotheses were addressed using the murine system and the MOG-induced active EAE model as a paradigm of CNS autoimmune inflammation.

5 Materials and Methods

5.1 Laboratory materials

5.1.1 Buffers, solutions and cell culture media

Solution	Components	Company
Phosphate buffered saline (PBS)	137 mM NaCl	Merck Millipore, Germany
	2.7 mM KCl	Carl Roth, Germany
	1.5 mM KH_2PO_4	Merck Millipore, Germany
	8.1 mM Na_2HPO_4	Sigma-Aldrich, Germany
	pH 7.2	
	or purchased from Gibco®	Thermo Fisher, USA
Complete medium	RPMI-1640	Gibco® Thermo Fisher
	10% Fetal calf serum (FCS)	Sigma-Aldrich
	100 U/ml penicillin	Biochrom, Merck Milipore
	100 µg/ml streptomycin	Biochrom, Merck Milipore
	1% Hepes 1M	Gibco® Thermo Fisher
	2 mM L-glutamin	Gibco® Thermo Fisher
	50 µM β-mercaptoethanol	Sigma-Aldrich
Wash medium	RPMI-1640	Gibco® Thermo Fisher
	5% FCS	Sigma-Aldrich
	100 U/ml penicillin	Biochrom, Merck Milipore
	100 µg/ml streptomycin	Biochrom, Merck Milipore
	1% Hepes 1M	Gibco® Thermo Fisher
FACS buffer	PBS	
	0.5% bovine serum albumin (BSA)	Serva, Germany
MACS buffer	PBS	
	0.5% BSA	Serva
	2 mM EDTA	Sigma-Aldrich

Cell fixing solution	PBS	
	4% paraformaldehyde (PFA) final concentration 2%	Carl Roth, Germany
Saponin solution	FACS buffer	
	0.5 % Saponin	Carl Roth
Red blood cell (RBC) lysis buffer	distilled water	
	150 mM KHCO3	Merck Millipore
	10 mM NH4Cl	Carl Roth
	0.1 mM Na2EDTA	Sigma-Aldrich
	pH 7.2 to 7.4	
MB solution	132 ml water	
	45 ml PBS 10X	Sigma-Aldrich
	3 ml 0.6N HCl	Carl Roth
	pH 7 to 7.2	
37% Percoll® solution for CNS cell isolation density gradient	Percoll® pH 8.5-9-5 MB solution	Sigma-Aldrich
37.5% Percoll® solution for liver cell isolation density gradient	Percoll® pH 8.5-9-5 MB solution 2mM EDTA	Sigma-Aldrich Sigma-Aldrich
Meninges digestion medium	Complete medium	
	2 mg/ml DNAse	Sigma-Aldrich
	2.8 mg/ml Collagenase type VIII	Sigma-Aldrich
Anaesthetic	415 mg/kg Ketamin	Iniesa Arzemittel, Germany
	9.7 mg/kg Xylazine in 0.9% NaCl solution	CP Pharma, Germany
Trypan Blue solution	PBS	
	0.4% (w/v) trypan blue	Sigma-Aldrich

5.1.2 Additional chemicals and reagents

Chemical / Reagent	Company
Brefeldine A	Biolegend, California, USA
Complete Freund's adjuvant (CFA)	Difco Laboratories, Detroit, USA
Dimethylsulfoxide (DMSO)	Sigma-Adlrich
Ethanol	Merck Millipore
Histopaque®	Sigma-Adlrich
Ionomycin	Sigma-Adlrich
IL-12, recombinant mouse	R&D Systems, Minneapolis, USA
IL-15, recombinant mouse	R&D Systems
IL-18, recombinant mouse	R&D Systems
*InVivo*Mab Anti-CXCR3 (clone CXCR3-173)	BioXcell, West Lebanon, USA
*InVivo*Mab polyclonal Armenian hamster IgG	BioXcell
Mycobacterium tuberculosis H37 RA	Difco Microbiology, Lawrence, USA
Myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55})	Pepceuticals Ltd., Leicester, UK
Pertussis toxin (PTX)	Sigma-Aldrich

5.1.3 Kits

Kit	Company
True-Nuclear™ Transcriotion Factor Buffer Set	Biolegend, California, USA
FoxP3 Transcription Factor Staining Buffer Set	eBioscience, Thermo Fisher
NK Cell Isolation Kit II, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany

5.1.4 Devices

Device	Company
Balance, Kern EW 820-2NM Benchtop	Kern & Sohn GmbH, Balingen Germany
Centrifuge	NeoLab, Heidelberg, Germany
Cell Incubator	Binder GmbH, Tuttlingen, Germany
Eppendorf Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Eppendorf Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
GFL WaterBath	GFL, Burgwedel, Germany
HeraCell Incubator	Heraeus, Hanau, Germany
HeraeBus Multifuge 3SR+	Thermo Fisher Scientific, Waltham, USA
Laminar flow hood HeraSafe	Heraeus, Hanau, Germany
LSR FortessaTM	BD Pharmigen, Heidelberg, Germany
MACS Separators and stand	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic stirrer	MLW RH3, Germany
Megafuge 1.0	Thermo Fisher Scientific, Waltham, USA
Neubauer counting chamber	Brand GmbH & Co KG, Wertheim, Germany
pH Meter	Schott Instruments GmbH, Mainz, Germany
Pipettes (2, 10, 20, 200 and 1000 µl)	Eppendorf AG, Hamburg, Germany
Pipetus®	Hirschmann Laborgeräte, Eberstadt, Germany
Rotamax 120 Orbital Shaker	Heidolph Instruments, Schwabach, Germany
Thermomixer Compact	Eppendorf AG, Hamburg, Germany
Timer	Eppendorf AG, Hamburg, Germany
Vacuum Pump	KNF Neuberger Inc., Trenton, USA
Vortex Genie 2	Scietific Industries Inc. Bohemia, USA
Wilovert S Inverted Microscope	Helmut Hund GmbH, Wetzlar, Germany

5.1.5 Software

Software	Company
BD FACSDiva™	BD Bioscience, Heidelberg, Germany
FlowJo v10.4	FlowJo LLC, Ashland, Oregon, USA
GraphPad Prism v8	GraphPad Software Inc., San Diego, USA
Mendeley®	Elsevier, Amsterdam, Netherlands

Microsoft Office 2016	Microsoft Corporation, Redmond, USA

5.1.6 General consumables

Consumable	Company
Cell strainers (75 and 100 µm pore size)	BD Bioscience, Heidelberg, Germany
Cellstar® serological pipettes	Greiner Bio-One GmbH, Frickenhausen, Germany
Corning® 12, 24, 48 and 96 -well culture plates	Sigma-Aldrich, Schnelldorf, Germany
Corning® Petri dishes	Sigma-Aldrich, Schnelldorf, Germany
Dumont medical dissection instruments: forceps, scissors, spatula, scalpels, hemostats and pins	Fine Science Tools GmbH, Heidelberg, Germany
Eppendorf tubes (0.5, 1.5 and 2 ml)	Eppendorf AG, Hamburg, Germany
Falcon™ conical tubes (15 and 50 ml)	BD Bioscience, Heidelberg, Germany
Falcon™ 5 ml round bottom tube with cell strainer cap	BD Bioscience, Heidelberg, Germany
MACS LS-Columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Micronic 1.10 ml tubes	Micronic, Lelystad, Netherlands
Parafilm	American Can Company, Greenwich, USA
Pipette tips (all sizes)	Sarstedt AG & Co, Nümbrecht, Germany
Reaction vessels (glassware)	Schott AG and DURAN Group GMbH, Maiz Germany
Reaction vessels (plasticware)	Vitlab, Großostheim, Germany
Serological pipettes	BD Medical, Heidelberg, Germany
Sterican® needles	B. Braun Melsungen AG, Hessen, Germany
Syringes	BD Bioscience, Heidelberg, Germany
Transfer pipette	Sarstedt AG & Co, Nümbrecht, Germany
TrueCount™ tubes	BD Bioscience, Heidelberg, Germany
Vasco® Nitril gloves	B. Braun Melsungen AG, Hessen, Germany

5.2 Mice

WT C57BL/6 mice were obtained from the Research Institute for Experimental Medicine (FEM) of the Charité (Berlin, Germany) and kept on a 12:12 h day:night cycle with *ad libitum* access to food and water. Rorc-Cre^{Tg}; Rosa26R$^{RFP/+}$ mice were provided by Chiara Romagnani. All animal experiments were approved by the regional animal study committee of Berlin (Landesamt für Gesundheit und Soziales) and performed in accordance to national and international guidelines.

5.3 *In vivo* methods

5.3.1 Induction and assessment of active EAE

Active EAE was induced by immunizing each mouse subcutaneously with 250 µg of myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) emulsified in complete Freund's adjuvant containing 800 µg of *Mycobacterium tuberculosis* H37Ra. Mice were immunized in the ventral flanks at four sites, in the axillar and inguinal areas, with a total volume of 50 µl per site. On the same day and after 48 hours, mice received an intraperitoneal injection of 200 µl of Pertussis toxin (PTX, 200 ng) dissolved in PBS. From the day 10 after immunization mice were weighted and examined daily for clinical symptoms and scored as follows: 0, no signs of clinical disease; 0.5, tail paresis; 1, tail plegia; 1.5, righting reflex weak; 2, hindlimb paresis; 2.5, unilateral hindlimb plegia; 3, bilateral hindlimb plegia; 3.5, forelimb paresis; 4, forelimb plegia. Mice were euthanized when they reached a score of > 3 or loss more than 20% of their weight in comparison with mice of their same strain and age. Mice used were always females of 10 to 14 weeks of age and were housed for at least one week in the animal facility before starting the immunization protocol.

5.3.2 Anti-CXCR3 antibody treatment

Mice were divided into two groups of 5 mice and immunized as stated in the previous section. The mice were then intraperitoneally injected with 200 µg of anti-CXCR3 antibody or control IgG diluted in 100 µl of sterile PBS on day 0, 3, 6, 9 and 12 post-immunization. Mice were sacrificed at day 15 post-immunization, and the organs were processed for flow cytometry analysis.

5.3.3 Mouse perfusion and organ removal

Mice were anesthetized with an intraperitoneal injection of a lethal dose of anesthetic containing xylazine and ketamine. When mice were deeply anesthetized, the pectoral cavity was opened starting from the abdominal cavity to expose the liver. The diaphragm was opened to expose the heart and the aorta was cut. The needle of a 26 G hypodermic lancet connected to a 12 cc syringe (20 ml) filled with PBS was inserted in the left ventricle of the heart, and the PBS was infused gently. In experiments where meninges and choroid plexus was collected, the mice were perfused with 60 ml of PBS by connecting the syringe to a 21 G butterfly needle that facilitates the exchange of syringes. After perfusion, the organs were removed. Only brains with no traces of blood were used in the experiments.

5.4 Isolation of immune cells from mouse tissue

All tissues were kept on ice (except blood) in tubes with medium until processed, no longer than one hour after dissection. The centrifugation steps were done at room temperature and without brake unless otherwise stated. The resulting cell solution was stored at 4°C until use for downstream applications, such as flow cytometry staining or *in vitro* culturing and assays.

5.4.1 Blood

Blood was collected from mice before perfusion after cutting the aorta. Blood was collected in tubes containing 500µl of 2 mM EDTA, mixed and kept at room temperature. Red blood cells were lysed with 10 ml of RBC lysis buffer for 10 minutes and centrifuged for 10 minutes at 300 g. The pellet was washed with 10 ml of wash medium, after centrifuging an additional time at 500 g for 5 minutes, the cells were resuspended in 1 ml of complete medium or FACS buffer and counted.

5.4.2 Spleen

The spleen was collected after perfusion by locating it at the dorsal left side of the mouse behind the liver. Each individual spleen was mashed over a 100 µm cell strainer placed over a 50 ml tube with a syringe plug, continuously washing the cell strainer with wash medium.

The tube was filled up to 20 ml of medium and centrifuged at 500 g for 5 minutes at 4°C. The pellet was resuspended in 10 ml of RBC lysis buffer and incubated for 10 minutes. 5 ml of mouse medium was added and then centrifuged for 10 minutes at 300 g. After that, the pellet was washed with 15 ml of wash medium, centrifuged for 5 min at 500 g, 4°C and resuspended in 10 ml of FACS buffer of complete medium to be counted.

In some experiments, the mononuclear cells of the spleen were isolated by gradient centrifugation. After mashing the spleen through the cell strainer and centrifuging, the cells were resuspended in 5 ml of complete medium, layered over 5 ml of Histopaque-1083 and centrifuged for 15 minutes at 800 g without brake. The layer of mononuclear cells was recovered between the medium and Histopaque layer and washed twice with complete medium.

5.4.3 Lymph nodes

The lymph nodes selected were the inguinal, axillary and brachial, unless stated otherwise. Lymph nodes were dissected after perfusion by separating the fur from the skin and locating them under the axillar area and over the microvasculature network in the inguinal area. The lymph nodes were mashed over a 100 µm cell strainer and washed with 10 ml of wash medium, after a centrifugation step at 500 g for 5 minutes at 4°C, the pellet was washed again with 10 ml of wash medium, centrifuged and resuspended in 5 ml of cmplete medium or FACS buffer for counting.

5.4.4 Liver

The liver was removed from the perfused mice and cleaned from the associated lymph nodes and gallbladder. The liver was then minced into small pieces and mashed through a 100 µm cell strainer with a syringe plug over a 50 ml tube. The tube was filled up to 50 ml with complete medium and centrifuged for 1 min at 60 g, without brake. The supernatant was transferred to a new 50 ml tube and centrifuged at 480 g for 8 min. The pellet was resuspended in a 10 ml of a 37.5% Percoll solution diluted in in MB medium containing 2mM EDTA in a 15 ml tube and centrifuged for 20 min at 850 g without brake. The resulting pellet was resuspended in 2ml of RBC lysis buffer and incubated for 5 minutes at room temperature. Afterwards, 5 ml of mouse medium was added and the solution was centrifuged at 480 g for 8 minutes at 8°C. The pellet was resuspended in 3 ml FACS buffer or complete medium and counted.

5.4.5 Central nervous system

The brain and the spinal cord was removed from the perfused mice. The tissue was mashed together through a 70 µm cell strainer washing extensively with complete medium until all tissue passed through the strainer. The solution was centrifuged at 400 g for 10 minutes at 4°C, the pellet was transferred to a 15 ml tube and resuspended in 6 ml of a 37% Percoll solution and centrifuged for 15 minutes at 2800 g without break. Afterwards, the myelin sheath that stayed in the upper layer of the Percoll solution was aspirated together with the Percoll. The pellet was recovered and washed two times in complete medium by centrifuging at 400 g for 10 min at 4°C. Between the first and the second wash, the cells were passed through a 30 µm filter to remove any residual myelin or connective tissue. Finally, the cells were resuspended in 1 ml of FACS buffer or mouse medium and stored at 4°C until use.

5.4.6 Meninges

The dural meninges were removed from the skullcap of the perfused mouse by carefully opening the skull from the ventral side of the head. For that, the skin, muscles, eyes and connective tissue was removed from the head; the jaw was removed by severing the mandibular junction and the skull was cut open by inserting the scissors in the auditory meatus and carefully cutting around the skull. The ventral side of the skull was discarded and the exposed brain was carefully scooped out with the spatula and kept for choroid plexus isolation. The remaining skullcap was placed in a small Petry dish with cold PBS under a dissecting microscope. The dural meninges was scored around the edge of the skull with the tip of a Dumont #5 forceps, scrapping also the membrane attached to the sutures of the skull. In that way, it was possible to peel off the complete meningeal tissue. The collected tissue was resuspended in 500 µl of PBS with 2 mg/ml of DNAse I and 2.8 mg/ml of Collagenase type VIII and digested at 37°C in a thermomixer for 20 minutes, shaking at 400 rpm. The digested meninges were then homogenized by pipetting a few times with a 1 ml pipette and passed through a 70 µm cell strainer, the cell strained was extensively washed with complete medium, and the solution was then centrifuged at 400 g for 7 minutes at 4°C. Finally, the pellet was resuspended in 1 ml of FACS buffer.

5.4.7 Choroid plexus

The choroid plexus was removed by placing the perfused brain with the dorsal side facing up and the caudal side (cerebellum) facing towards the examiner in a Petry dish with cold mouse medium, under a dissecting microscope. The cerebellum was opened coronally in the junction with the cerebrum with a scalpel to access the fourth ventricle. The choroid plexus was removed from the cavity of the fourth ventricle with fine forceps and transferred to a tube with cold PBS. The choroid plexus from the third ventricle was removed by doing an incision with the scalpel starting at the confluence of the sinuses toward the sagittal sinus, trespassing the corpus callosum until the third ventricle was reached. The choroid plexus was then found attached to the upper wall of the ventricle. Finally, the lateral ventricles were exposed by cutting sagittaly with the scalpel about 4 mm parallel to the middle sinus on each side until the ventricles were exposed. The choroid plexuses were located hanging in the ventricle attached to the lateral walls and to the floor of the ventricles. The choroid plexus was then mashed through a 70 µm cell strainer extensively washing with PBS containing 5 mM EDTA. The solution was centrifuged 10 minutes at 600 g, at 4°C and the pellet resuspended in 1 ml of FACS buffer.

5.5 Cell sorting methods

5.5.1 Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) was used to separate NK cells from a preparation of mouse lymph nodes and/or splenocytes. The MACS technology consists on the use of a magnetic field to retain cells labeled with magnetic microbeads in a column attached to a magnet. The kit used in this study is based on negative selection strategy to isolate NK cells. Hereby, the non-target cells are magnetically labeled and retained in the column, while the unlabelled NK cells pass through the column and are collected. The protocol was followed according to the manufacturer's instructions working with pre-cooled solutions and centrifuge to avoid unspecific labeling. Briefly, cells were resuspended in MACS buffer (PBS + 0.5% BSA + 2mM EDTA, pH 7.2) and incubated for 5 minutes with a Biotin-Antibody Cocktail containing biotin-conjugated monoclonal antibodies against antigens not expressed on NK cells. Next, the cells were incubated for 10 minutes with a MicroBead Cocktail containing magnetic microbeads coupled to anti-biotin antibodies. The cells were then passed through a LS Separation Column attached to a midiMACS magnet and collected, the column was washed three times with 3ml of MACS buffer. Finally, the collected NK cells were centrifuged for 10 minutes at 400 g, resuspended in 3 ml of complete medium and counted.

5.5.2 Flow activated cell sorting

Flow activated cell sorting (FACS) uses the technology of flow cytometry (explained in section 6.7) to separate cells based on the phenotypic characteristics previously specified by the examiner. In brief, a heterogeneous mixture of cells is stained with fluorescent antibodies that target the markers of interest. The cells are then analyzed in the flow cytometer to identify the populations of interest. The selected populations are separated by using a vibrating mechanism to distribute each cell in electrically charged single droplets; the droplets that contain the desired cell type are then directed into a collection tube by using electrostatic attraction.

The separation of NK cell subsets using FACS was performed by the team members of the flow cytometry lab of the BCRT, using a BD FACSAria II cell sorter. Before sorting, cells from the lymph nodes and spleen were isolated with Histopaque gradient centrifugation (see section 6.4.2) and stained up to 50 x 10^6 cells in a total volume of 200 µl as described in section 6.7.2. The stained cells were handed to the Flow Cytometry lab and the separated cells were returned in 1 ml tubes with FACS buffer.

5.6 *In vitro* assays

5.6.1 Cell culture

In some experiments, immune cells obtained from mouse tissue were cultivated before or after cell sorting for hours to days under physiological conditions. These experiments were performed under a laminar flow hood under aseptic conditions. The tools and material used in the hood were previously sterilized by autoclaving them at 120°C, 1 bar when possible and disinfecting them with 70% ethanol. In addition, sterile buffers, cell culture plates, pipettes and tubes were purchased. The cells were cultured in complete medium, in an incubator at 37°C with 5% CO2 and 95% humidity.

5.6.2 Cell counting

To determine the number of live cells in a suspension, the cells were mixed with Trypan blue, a dye that enters through the damaged membrane when cells die. In this way, the blue colored dead cells can be distinguished from the live bright cells. Cells were counted in a Neubauer chamber that contains visible quadrants where the cells lie. A cell sample

suspended in a defined volume (V) were diluted in a defined ratio with trypan blue (usually 1:10). Ten µl of the mix was applied to the chamber and the cells were counted under the microscope with a manual counter in at least two quadrants. The average of the cells in one of the quadrant was obtained (n) and the total number was calculated with the following formula:

Total number of cells = n x trypan blue dilution factor x V x 10 000 (chamber factor)

5.6.3 Stimulation of immune cells

Splenocytes, lymph node cells, immune cells isolated from the CNS or MACS/FACS isolated NK cells were stimulated *in vitro* in round bottom 96 well plates in a concentration of 100 000 cells per well, or in 48 well plates in a concentration of 10^6 cells per well. To test the production of cytokines, cells were stimulated in complete medium with either 50 ng/ml of PMA and 5 µg/ml of Ionomycin, or with 5 ng/ml of IL-12 and 50 ng/ml of IL-18 in the presence of the protein transport inhibitor Brefeldin A (10µg/ml) for 5 hours at 37°C. As a control, cells were cultured with Brefeldin A without stimulants. In CXCL10 assays, cells were incubated with different concentrations of CXCL10 in complete medium in the presence of IL-15 (50 ng/ml) when incubated for more than 4 hours. After stimulation, the cells were washed with complete medium (centrifuged at 400 g for 7 minutes) for further analysis.

5.7 Flow cytometry

Flow cytometry is the most used technology to analyze physical and chemical characteristics of thousands of individual cells in a “real time” manner. This laser-based technology incorporates the use of a hydrodynamic flow that focuses a cell suspension into a single cell stream that passes through a beam of light. The light scattered by each cell as it passes through the beam is measured in two angles to determine the physical characteristics of the cell such as size and internal complexity. The forward scatter detector (FSC) is at the opposite side of the ray of light and determines the relative size of the cells, while the sideward scatter detector (SSC) located perpendicular to the ray of light, provides information about the granularity of the cells. Information about the size and granularity is useful to distinguish among different populations of cells in a sample, for example, NK cells are part of the lymphocyte population, which is small and have low granularity compared with the neutrophils, which are larger and have multilobed nucleus and numerous granules in the cytoplasm. The flow cytometer is also equipped with laser beams of specific wavelengths that excite fluorescent

dyes or flourochromes coupled to antibodies that target surface or intracellular components of the cells. The fluorescence emitted passes through special arrays of filters to guide a determined wavelength to the sensors that detect fluorescence at specified wavelengths, called photomultiplying tubes (PMTs). The PMTs convert the light signal into voltage and amplify it to finally be detected and analyzed by a computer workstation (208). New cytometers such as the Cytoflex from Beckman Coulter incorporate avalanche photo diodes detectors instead of PMTs to detect the fluorescence signals.

The emission spectra of some fluorochromes partly overlap; as a consequence, one fluorochrome can be detected in more than one PMT leading to false positive signals. Therefore, and automatic or manual compensation has to be done to subtract the spillover signal.

The flow cytometer used in the current study was a BD Fortessa™ coupled to the acquisition software FACSDiva™. Compensation was done using single stained cells or beads in the FlowJo software version 10.4. Flow cytometry analysis was also done in the FlowJo software. Fluorescence minus one controls were used to define the gate of the positive population.

5.7.1 List of antibodies and reagents used for flow cytometry

Each antibody was titrated in using cells with a known expression of the marker to determine the optimal working dilution. The antibodies purchased were manufactured to target mouse proteins.

Target	Fluorochrome	Clone	Dilution	Company
CD3	PacBlue	500A2	1:100	BD Biosciences
	FITC	145-2C11	1:100	BD Biosciences
CD11b	APC-Cy7	M1/70	1:50	BD Biosciences
CD16/CD32 (Fc Block)	-	2.4G2	1:50	BD Biosciences
CD19	Biotinylated	6D5	1:200	Biolegend
CD27	Biotinylated	LG.7F9	1:200	eBioscience
	PE-Cy7	LG.7F9	1:50	eBioscience
CD45	FITC	30-F11	1:200	eBioscience
CD49a	PE-Vio770	REA493	1:50	Miltenyi Biotec

CD49b	Biotinylated	DX5	1:200	Biolegend
	PE-Dazzle596	DX5	1:100	Biolegend
CD69	PerCP-Vio700	HI.2F3	1:10	Miltenyi Biotec
CD90.2 (Thy1.2)	AF-700	30-H12	1:200	Biolegend
CD103	Biotinylated	2E7	1:200	Biolegend
CD127 (IL-7Rα)	PE	A7R34	1:50	Biolegend
CD200R	PE	OX-110	1:200	Biolegend
CCR2	AF-647	SA203G11	1:100	Biolegend
CCR7	AF- 488	4B12	1:100	eBioscience
cKit	Brilliant Violet-605	ACK2	1:100	Biolegend
CXCR3	Brilliant Violet -510	CXCR3-173	1:50	Biolegend
CXCR4	PE	2B11	1:100	BD Biosciences
CXCR6	PE	DANID2	1:50	eBioscience
CX3CR1	PerCP-Cy5.5	SA011F11	1:50	Biolegend
DNAM-1	PE	480.1	1:200	Biolegend
Eomes	PEeF610	Dan11mag	1:50	Biolegend
FcεRIα	Biotinylated	MAR-1	1:200	Biolegend
Gr-1	Biotinylated	RB6-8C5	1:200	Biolegend
IFN-γ	PE	XMG1.2	1:25	eBioscience
Ki67	PerCP-eF700	SolA15	1:1000	eBioscience
NK1.1	APC	PK136	1:50	Miltenyi Biotec
NKp46	PE	29A1.4.9	1:50	Miltenyi Biotec
Perforin	PE	S16009A	1:500	Biolegend
RORγt	BV786	Q31-378	1:50	BD Biosciences
	PE	Q31-378	1:800	BD Biosciences
Streptavidin	Brilliant Violet-650	-	1:400	Biolegend
Tbet	Brilliant Violet-711	4B10	1:50	Biolegend
	PE-Vio-615	REA102	1:30	Miltenyi Biotec
TNF-α	PerCP-Cy5.5	MP6-xt22	1:150	eBioscience
TRAIL	Biotinylated	N2B2	1:100	Biolegend

5.7.2 Staining of cells for flow cytometry

Up to 3 x 10^6 cells were stained in 1.10 ml Micronic tubes in in a total volume of 50 µl of FACS buffer at 4°C. First, cells were incubated for 15 minutes with 25 µl of anti-mouse CD16/CD32 (Fc-Block) to block the Fc-Receptors. Afterwards, a cocktail of monoclonal anti-mouse antibodies targeting surface markers were added and incubated for 20 minutes. When biotinylated antibodies were used, the cells were washed with 1 ml of FACS buffer, centrifuged at 400 g for 10 min, resuspended in FACS buffer containing fluorophore-coupled streptavidin and incubated for 15 minutes at 4°C. The cells were finally washed with 1 ml of FACS buffer, centrifuged, resuspended in 100-200µl of FACS buffer and transferred to (FACS) tubes to be analyzed in the flow cytometer. Each sample was filtered through a 30 µm pore filter cap before placing it in the flow cytometer.

For intracellular staining, the cells were washed with PBS after surface staining and resuspended in 2% paraformaldehyde (PFA) diluted in PBS to fix them during 20 minutes at room temperature in the dark. Next, the cells were washed with 800 µl of saponin buffer (0.5% saponin diluted in FACS buffer) to permeabilze the cell membrane. The cells were then stained for 20 minutes at 4°C with the antibody mix diluted in saponin buffer. The cells were finally washed with 1 ml of saponin buffer and resuspended in PBS to be analysed in the flow cytometer.

For intranuclear staining, the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set was used to fix and permeabilize the cells according to the manufacturer's instructions. This kit was also used to stain intracellular targets together with intranuclear targets.

5.8 Statistics

Two-tailed, parametric T-test was used for two-group comparisons. Gaussian distribution of the variables was confirmed with the Kolmogorov-Smirnov normality test. Some groups failed to pass the normality test due to the presence of an outlier, in that cases a parametric test was used nonetheless. For non-normally distributed variables, a non-parametric Mann-Whitney test was used instead. A p value <0.05 was considered significant and was depicted as *= $p<0.05$, **=$p<0.01$ and ***=$p<0.001$. All statistical analysis was performed in GraphPad Prism 5 and 8.

6 Results

6.1 Characterization of the chemokine receptors of immature NK cells in the CNS

6.1.1 CX3CR1 mediate the recruitment of mature NK cells to the CNS during neuroinflammation

Throughout the first year of my PhD, I focused together with my colleague L. Hertwig in defining the chemokine tools that aids on the migration of mature NK cells into the CNS. We made use of the transgenic mice deficient on the fraktalkine (CX3CL1) receptor, the CX3CR1, on the C57Bl/6 background. In these mice, the CX3CR1 locus was substituted by a reporter green fluorescent protein (GFP) and is interchangeably referred as $CX3CR1^{GFP/GFP}$ or $CX3CR1^{-/-}$ mice (Huang 2006). To evaluate the NK cell subsets that are recruited into the CNS during neuroinflammation, EAE was induced in these mice. We found that $CX3CR1^{-/-}$ mice have a reduced migration of mature NK cells into the CNS compared to the WT mice (Figure 1A). We then performed adoptive transfer experiments in which $CX3CR1^{-/-}$ recipient mice were transferred intravenously with isolated NK cells (0.5 – 1 x 10^6 cell per mouse) derived from WT or $CX3CR1^{-/-}$ mice, or PBS as a control. With these experiments, we showed that the mice that received WT ($CX3CR1^{+/+}$) NK cells had a higher proportion of mature NK cells in the CNS at day 20 post immunization than the mice that received CX3CR1-deficient NK cells (Figure 1B). This was accompanied with a reduction on the EAE severity (Figure 1C), indicating a protective role of mature NK cells during neuroinflammation. The figure 1 summarizes these findings. The complete data is available in the published manuscript which I co-authored in: Hertwig L, Hamann I, Romero-Suarez S, et al. CX3CR1-dependent recruitment of mature NK cells into the central nervous system contributes to control autoimmune neuroinflammation. *Eur J Immunol*. 2016;46(8):1984-1996.

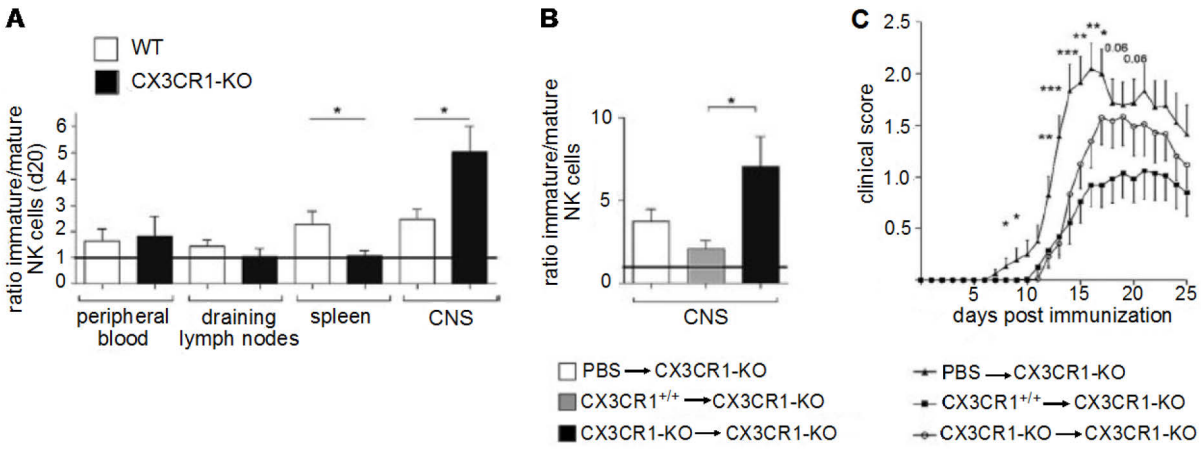

Figure 1. CX3CR1-dependent recruitment of mature NK cells into de inflamed CNS. A) EAE was induced in WT and CX3CR1$^{-/-}$ mice (n = 10 and n = 8 per group, respectively, from three independent experiments) and the NK cell maturation subsets were analysed by flow cytometry in the different organs at day 20 post immunization (p.i.). The ratio of immature (CD11b-) to mature (CD11b+) NK cells is shown. B) NK cells isolated from WT and CX3CR1$^{-/-}$ mice were transferred intravenously into CX3CR1$^{-/-}$ mice one day before EAE induction (n = 9 and n =5 per group, respectively, from three independent experiments). PBS injection served as a control (n = 9). The ratio of immature to mature NK cells in the CNS at day 20 p.i. is shown. C) Clinical EAE course after the adoptive transfer of WT or CX3CR1$^{-/-}$ NK cells or PBS into CX3CR1$^{-/-}$ mice (n = 24 per group, performed in 6 independent experiments). Graphs show mean ± standard error of the mean (SEM). *p<0.05; nonparametric Mann-Whitney test. Clinical scores were analysed using the Kruskal-Wallis test (with Dunn's posthoc test) *p<0.05, **p<0.01, ***p<0.001. Figure adapted from Hertwig *et al.* 2016.

6.1.2 Immature NK cells are present in the healthy and inflamed murine CNS

Thereafter, I set out to investigate whether immature NK cells were present in the murine CNS during neuroinflammation. To identify NK cells and their maturation subsets in the CNS by flow cytometry, a quality control gating strategy to exclude duplets and dead cells with 7AAD, which stain cells with a disrupted membrane, was always performed as seen in Figure 2A. Mononuclear cells were identified in the forward vs sideward scatter and from there, the NK cells were gated as CD45^{high}CD11b$^{low/-}$CD3-NK1.1+ cells. This gating strategy excluded potentially contaminating cells such as B cells (identified by CD19), granulocytes (identified by Gr-1) and eosinophils, mast cells and basophils (identified by FcεRIα). Furthermore, all NK1.1+ cells were NKp46+ (Figure 2D, solid line). The maturation subsets of NK cells were identified with the markers CD27 and CD11b. Figure 2B shows the gating strategy in the naïve and EAE mice. As expected, the EAE mice had a higher density of infiltrating cells including monocytes, CD3+ T cells and CD3- cells, as well as NK cells. Interestingly, the NK cells in both the naïve and the EAE mice comprised all the maturation stages with a significant decrease in immature NK cells during EAE (Fig. 2C).

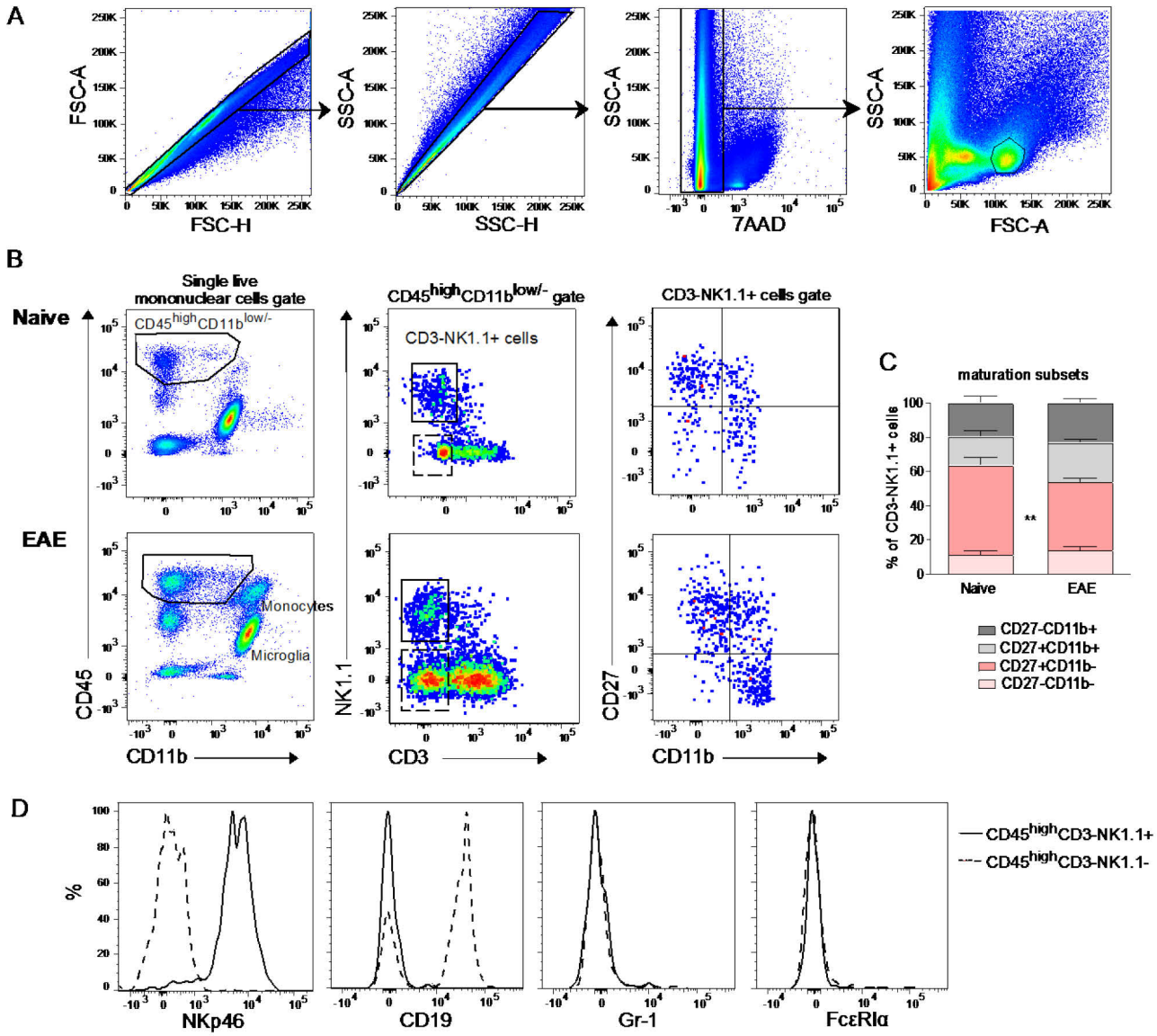

Figure 2. NK cell maturation subsets in the healthy and inflamed CNS. Single cell suspension from murine CNS were stained and analyzed by flow cytometry. A) Representative pseudocolor dot plots showing the general gating strategy to select single, live and mononuclear cells in CNS samples. B) Representative dot plots showing the gating strategy to detect NK cells and its maturation subsets in the naïve and the EAE sick mouse. C) Stacked graph of the frequency of each NK cell maturation subset from the NK cell gate (CD3-NK1.1+) in the naïve (n=10) and EAE mice (n=20). Data represents 4 independent experiments and were analyzed with an unpaired t-test **p<0.01 D) Histogram overlay showing the fluorescence intensity of the expression of the indicated markers in the NK cell population (solid line) comparing with a non-NK cell gate (dotted line, gate shown in B). Representative figure of 6 naïve mice.

6.1.3 Immature NK cells in the CNS express CXCR3

To better characterize the immature NK cells that are present in the CNS, the expression of the chemokine receptors that might aid in the migration of this subset into the CNS was evaluated in unmanipulated mice. Based on the known expression of the ligands in the CNS (130) and on previous reports of chemokine receptors on NK cell subsets in both mice and humans (48,209–211), the chemokine receptors CXCR3, CXCR4, CCR2, and CCR7 were selected as candidates to be expressed in immature NK cells. First, the expression of the chemokine receptors on the NK cells present in the healthy CNS was analyzed by flow cytometry. A big proportion of NK cells expressed CXCR3. In contrast, less than 2% of NK cells expressed CXCR4, CCR2 and CCR7 (Fig. 3A). CXCR3 was expressed also in the peripheral organs (Fig. 3B) and was specifically expressed in immature NK cells in the CNS and the periphery (Fig. 3C). The highest proportion of CXCR3+ NK cells was found in the lymph nodes, which is in line with the known enrichment of immature NK cells in this organ.

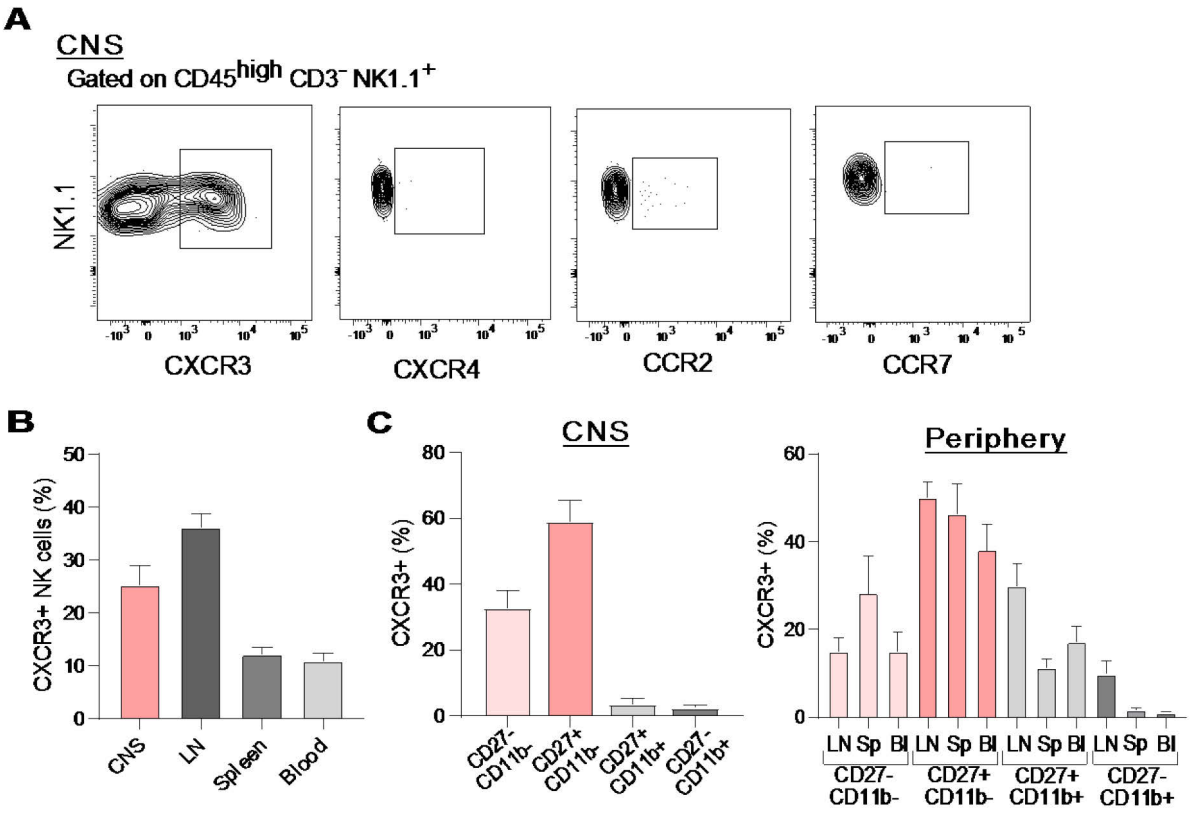

Figure 3. Chemokine receptors on immature NK cells in the CNS and the periphery A) Representative contour plots of the expression of the indicated chemokine receptors plotted against NK1.1 on the CNS of naïve mice. B) Graph showing the frequency of CXCR3+ NK cells in the indicated organs (n: CNS = 18; LN, Sp, Bl = 12). C) Graph showing the proportion of CXCR3+ cells on the indicated maturation subsets of NK cells in the CNS (n= 10) and in the periphery n ≥ 5. LN, lymph nodes; Sp, spleen; Bl, blood.

6.1.4 CXCR3+ NK cells decrease in the CNS during EAE

As some chemokine receptors are upregulated during inflammation after cell activation, the proportion of NK cells expressing the above-mentioned chemokine receptors in the CNS of EAE mice was evaluated. There was no change in the expression of CXCR4, CCR2 and CCR7 in the NK cells present in the inflamed CNS; however, there was a dramatic decrease in the proportion of CXCR3+ NK cells in the CNS of EAE mice.

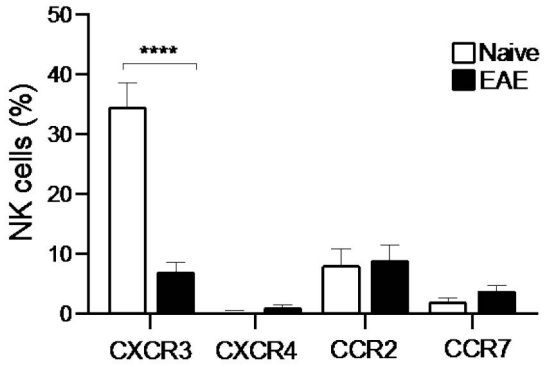

Figure 4. Expression of chemokine receptors on NK cells during EAE. Graph showing the proportion of NK cells expressing the indicated chemokine receptors in the CNS of naïve and EAE sick mice analyzed by flow cytometry. N: CXCR3 Naïve= 13, EAE= 20 performed in 4 independent experiments; CXCR4, CCR2 and CCR7 = 4 mice per group, performed in 2 independent experiments. The difference between the naïve and EAE group was analyzed with an unpaired t-test, ****p<0.0001.

6.1.5 The surface expression of CXCR3 on NK cells decreases after activation with CXCL10

The ligands of CXCR3 – CXCL9, CXCL10 and CXCL11 – are induced by IFN-γ and hence upregulated in the CNS during MS and EAE (212,213). Thus, I reasoned that the downregulation of CXCR3 on NK during neuroinflammation might be due to an increased CXCR3 activation by its ligands, which provokes an internalization of the receptor, as has been previously reported in T cells (214–216). To test whether this was also the case in NK cells, an *in vitro* assay with CXCL10 was performed. Lymphocytes isolated from the lymph nodes (axillar and subiliac lymph nodes), which has the highest proportion of immature CXCR3+ NK cells, were cultured together with increasing concentrations of CXCL10 in complete medium for 4 hours and the expression of CXCR3 on NK cells was analyzed by flow cytometry. Figure 5A shows the gating strategy to identify NK cells and CXCR3 in fresh cells isolated from lymph nodes. After incubation with CXCL10, the expression of CXCR3 decreased in a dose dependent manner as shown in Fig.5B.

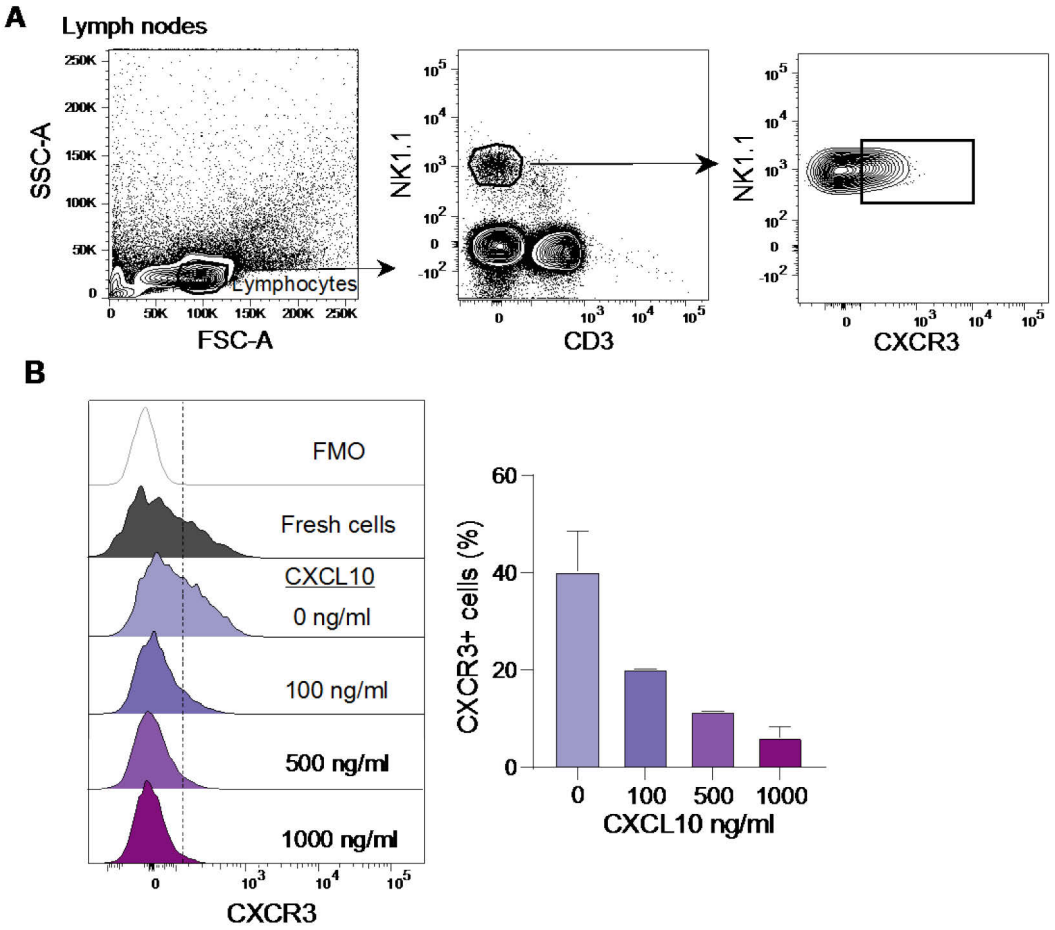

Figure 5. Surface expression of CXCR3 after CXCL10 ligation. Cells where isolated from the lymph nodes of naïve mice and cultured in complete medium for 4 hours in the presence of increasing concentration of CXCL10. A) Representative contour plots showing the gating strategy to detect CXCR3+ cells in isolated lymph node cell preparation. B) Overlaid histograms showing the fluorescence intensity of CXCR3 expression on fresh NK cells from fresh LN cells and after 4 hours of incubation with the indicated concentrations of CXCL10. The percentage of CXCR3+ cells is plotted at the right. The CXCR3+ gate was defined with a fluorescence minus one control (FMO) as seen in the histogram overlay. Data from two independent experiments.

6.1.6 Activation of CXCR3+ NK cells with CXCL10 does not induce NK cell maturation nor IFN-γ secretion

It has been reported that the activation of CXCR3 with CXCL10 induces the polarization of activated T cells into Th1 IFN-γ producing cells (217). Based on that, it was explored whether the signaling through CXCR3 in NK cells influence their maturation status and IFN-γ production. First, an in vitro assay using a high concentration of CXCL10 (500 ng/ml) in isolated NK cells from spleen and lymph nodes was performed. After 4 hours of incubation, the maturation status of NK cells remained unchanged (Fig. 6A). The assay was then performed with FACS sorted CXCR3+ NK cells from spleen and lymph nodes incubated for 48h with a

lower concentration of CXCL10 (100 ng/ml), as in Zohar 2014. The culture was supplemented with 50 ng/ml of IL-15 to support the survival of NK cells (Fig. 6B). As a positive control, cells were stimulated with IL-12 and IL-18, which induced NK cell maturation and IFN-γ secretion. Again, the long-term stimulation of CXCR3+ NK cells with CXL10 did not induce NK cell maturation or IFN-γ secretion (Fig. 6C).

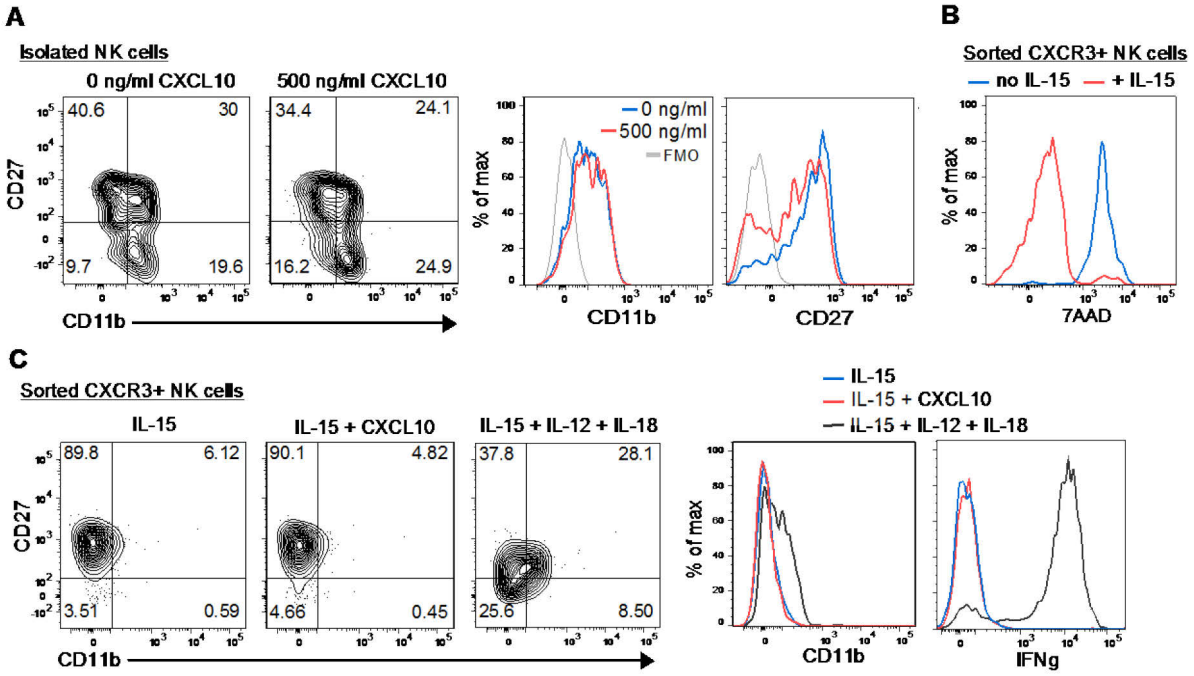

Figure 6. Maturation of NK cells after short and long term incubation with CXCL10. A) NK cells from the spleen and lymph nodes were isolated by untouched magnetic cell sorting (MACS) and incubated for 4 hours in complete medium in the absence or presence of 500 ng/ml CXCL10. The maturation stages were then analysed by flow cytometry. Contour plots show the mean percentage of each population, at the right, overlaid histogram show the fluorescence intensity of CD11b and CD27 of the incubated NK cells. Representative figures from 3 independent experiments. B) Overlaid histogram of the fluorescence intensity of 7AAD after 48h incubation of sorted CXCR3+ NK cells in complete medium with or without 50 ng/ml of IL-15. C) Sorted CXCR3+ NK cells were incubated for 48 hours in complete medium supplemented with IL-15 (50 ng/ml) in the presence of CXCL10 (100 ng/ml) or IL-12 (5 ng/ml) and IL-18 (10 ng/ml). The maturation status and intracellular IFN-γ was analysed by show cytometry. For IFN-γ analysis, Brefeldine A (10 µg/ml) was added to the culture for the last 5 hours of incubation. Representative contour plots and overlaid histograms of 3 independent experiments.

6.1.7 Anti-CXCR3 antibody treatment does not alter the proportion of immature NK cells in the CNS of EAE mice

After confirming that immature NK cells that express CXCR3 downregulate their receptor surface expression after CXCL10 ligation, without altering their maturation and activation status, a pilot experiment to explore whether CXCR3 was involved in the recruitment of immature NK cells into the CNS during neuroinflammation was performed. For that, mice were

treated with five alternating (every third day) intraperitoneal (i.p.) injections of a blocking CXCR3 antibody or a IgG isotype as a control (5 mice per group), starting on the day of immunization. The mice were sacrificed 3 days after the last injection (day 15), at a time when mice normally show clinical disease. Figure 7A shows a scheme of the experimental design.

The presence of NK cells and its maturation stages were subsequently compared between the groups in the brain, spinal cord (SC), lymph nodes (LN), spleen and blood by flow cytometry (Fig. 7B, C). Unfortunately, 3 of the mice in the anti-CXCR3 group and 2 of the isotype control group did not survive the treatment regimen due to complications from the repeated i.p. injections. Of note, only one mouse in the control group showed signals of clinical disease at the day of sacrifice. There was no change in the proportion or numbers of NK cells in the different organs between the anti-CXCR3 and control group (Fig. 7B). In addition, the maturation subsets were unchanged between the groups in all the organs except in the spleen, where a lower proportion of immature (DN: CD27-CD11b- and CD27+CD11b-) and a higher proportion of mature (CD27-CD11b+) NK cells was found in the mice treated with anti-CXCR3 antibody (Fig 7C). Despite the low power of the experiment, the results suggest that CXCR3 is important for the homing of immature NK cells into the spleen during inflammation. A higher-powered experiment would be necessary to exclude the involvement of CXCR3 in the recruitment of NK cells into the CNS.

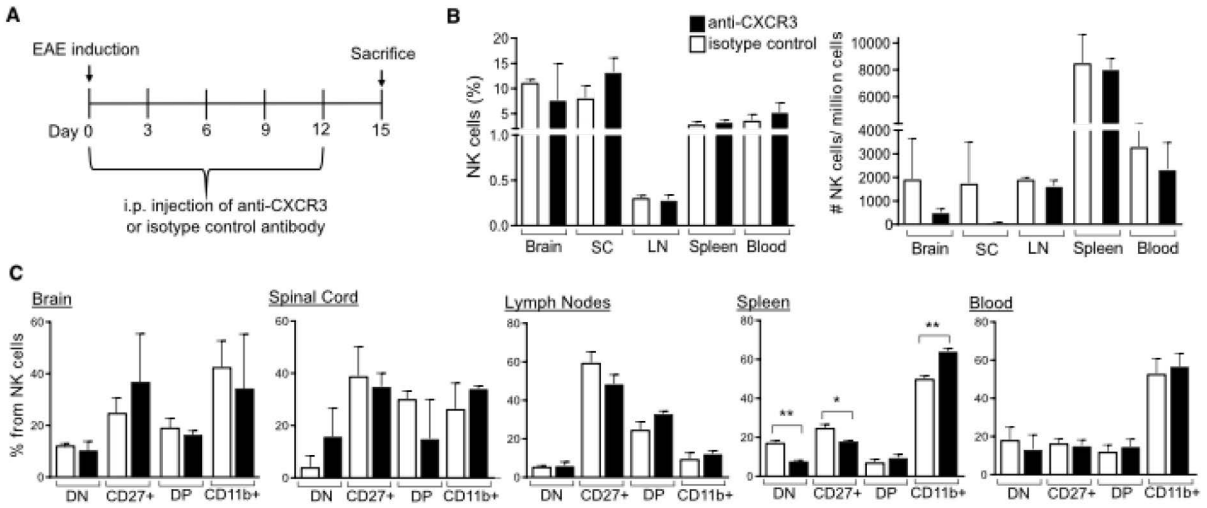

Figure 7. Effect of anti-CXCR3 treatment during EAE on the recruitment of NK cells into the CNS and periphery. A) EAE was induced and the mice received five i.p injections of anti-CXCR3 or control isotype IgG (200 µg diluted in PBS, each) every third day as shown in the scheme. B) Graphs showing the percentage and numbers of NK cells in each organ determined by flow cytometry. C) Graphs showing the percentage of each maturation subset from the NK cell gate in each organ analyzed by flow cytometry. N: anti-CXCR3 = 2, isotype control = 3. Graphs show mean + SEM. The groups were analyzed with an unpaired t-test *p<0.05, **p<0.01. SC, spinal cord; LN, lymph nodes; DN, double negative CD27-CD11b-; DP, double positive CD27+CD11b+.

6.2 Identification of different ILC group 1 subsets in the CNS

The presence of NK cells in the CNS during steady state suggest the possibility that some of them might represent tissue resident cells, i.e. ILCs. Furthermore, the expression of CD27 and CXCR3 has been reported in liver ILC1s (17,56,61), which supports the idea of a ILC1 population on the CNS that express 'immature NK cell' markers. Therefore, I decided to characterize the CNS CD3-NK1.1+ cells in the new ILC frame and include markers that would provide more detail about their true identity.

6.2.1 Immature NK cells express the ILC1 marker CD49a

The markers CD49a and CD49b were used to distinguish between ILC1 and conventional NK cells, respectively. We gated on the conventionally identified NK cell maturation subsets as shown in Figure 1 and analysed the expression of CD49a and CD49b within the maturation subsets excluding the DN subset due to the low number of cells. More than half of the immature CD27+CD11b- NK cells expressed CD49a and lacked the expression of CD49b. On the other hand, the mature (CD27+CD11b+) and terminally mature (CD27-CD11b+) NK cells were mostly CD49a-CD49b+ (Figure 8). This data suggest that a considerable fraction of the cells previously recognized as immature NK cells present in the healthy CNS are not conventional NK cells but ILC1s.

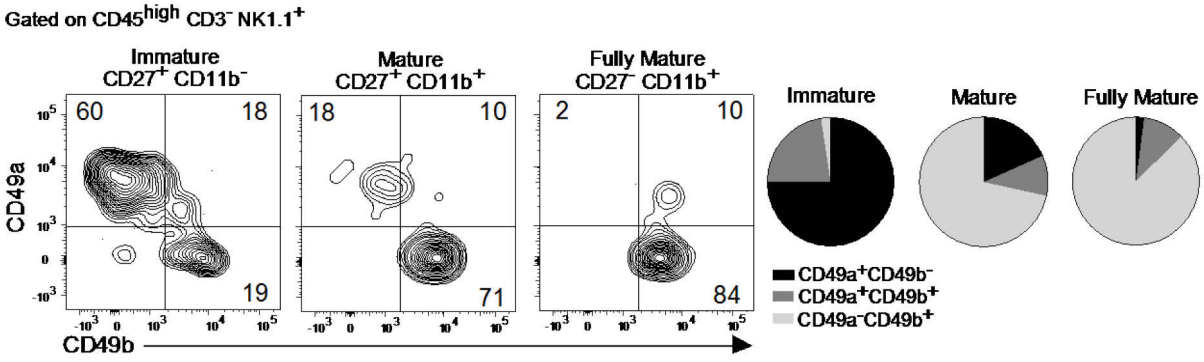

Figure 8. Expression of CD49a and CD49b in the NK cell maturation subsets. Representative contour plots showing the expression of CD49a and CD49b in the different NK cell subsets of the CNS of naïve mice. The numbers indicate the mean percentage of each subset of n = 6. At the right, a visualization in pie charts show the proportion of the CD49a+CD49b-, CD49a+CD49b+ and CD49a-CD49b+ populations within the NK cell maturation subsets.

6.2.2 CD3-NK1.1+ CNS cells comprise diverse ILC populations

Next, the proportion of the subsets identified by the integrins CD49a and CD49b were assessed directly in the ($CD45^{high}CD11b^{low/-}$)CD3-NK1.1+ cells of the CNS comparing with the liver, which is known to contain both conventional NK cells and ILC1s, and the spleen, which is known to contain NK cells but very little ILC1s. Similar to the liver, about 30% of CNS CD3-NK1.1+ cells were CD49a+CD49b- while about 60% were CD49a-CD49b+; in addition, about 10% of the CD3-NK1.1+ cells expressed both CD49a and CD49b (Fig. 9A). As expected, most CD3-NK1.1+ cells of the spleen were CD49b+ (Fig 9A).

To confirm the identity of the identified subsets we performed flow cytometry analysis of the transcription factors Eomes, Tbet and RORγt. The CD49a-CD49b+ subset expressed Tbet and Eomes, confirming their NK cell identity; while CD49a+CD49b- cells were Tbet+Eomes-, confirming their ILC1 identity. Interestingly, within the CD49a+CD49b+ subset, about half were Eomes+ and half Eomes-, which could represent an intermediate stage between NK and ILC1 cells as has been previously reported (68). Therefore, this population was designated intermediate (int) ILC1s. Furthermore, none of the subsets contained ILC3s as they did not express RORγt (Fig. 9B). However, it is known that ILC3s can differentiate into ILC1 by decreasing RORγt after IL-12 stimulation. Therefore, to determine whether some of the ILC1s had an ILC3 origin (i.e. ex-ILC3s) the genetic fate mapping (fm) mouse for RORγt (Rorc-Cre^{Tg};$Rosa26R^{RFP/}$+, referred as $RORc^{fm}$) was used. In this mouse, all the cells that ever expressed RORγt (Rorc gene) are permanently and heritably marked with the red fluorescent protein (RFP), even if they subsequently lose RORγt expression (93). Interestingly, about 35% of both the ILC1s and intILC1s were ex-ILC3s (34.6 ± 4.4 and 35.7 ± 7.2 %, respectively), while less than 3% of the NK cells were RFP+ (2.9 ± 1.4 %) (Fig. 9C).

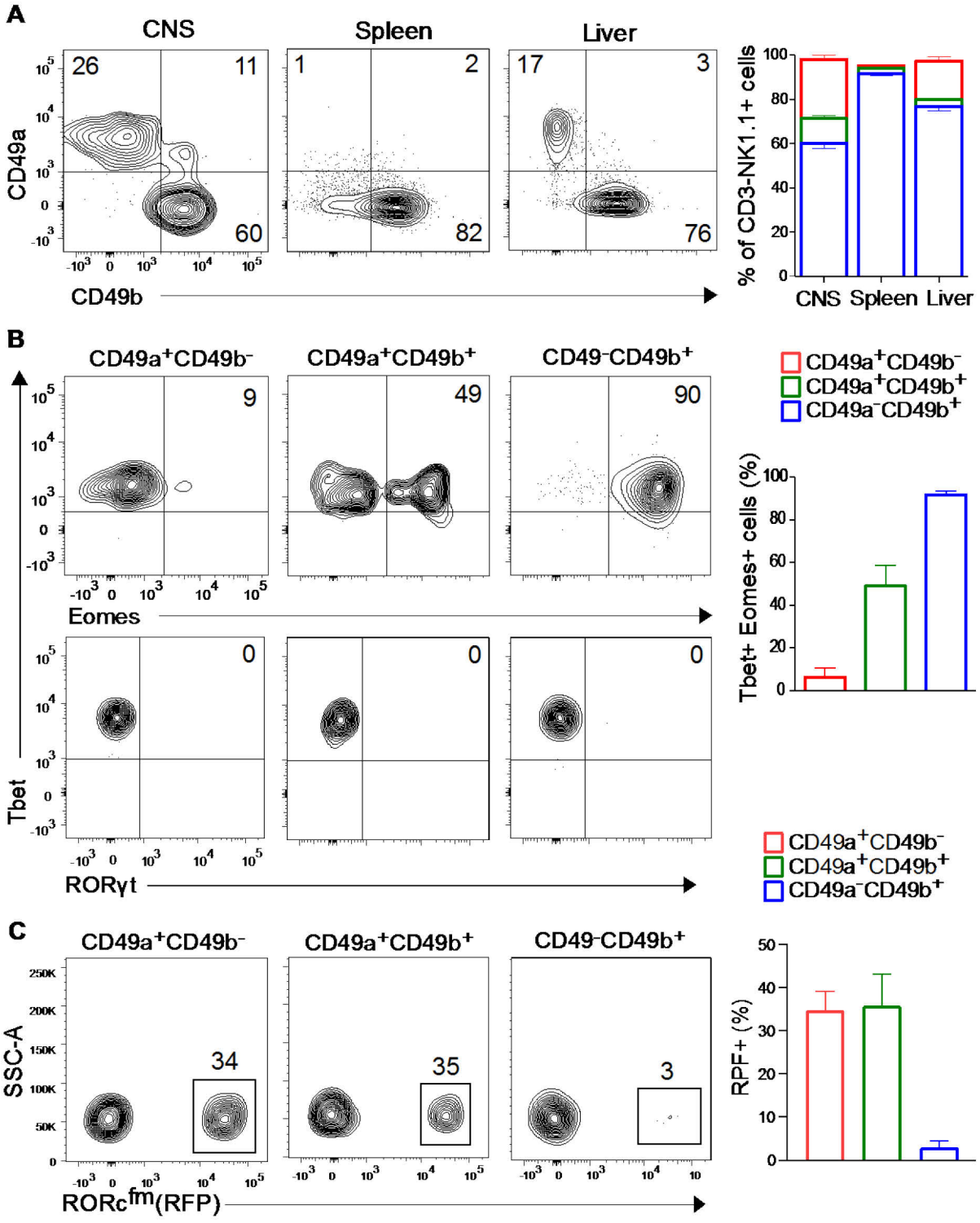

Figure 9. Identity of the NK1.1+ CNS populations defined by the expression of transcription factors. CD45^{high}CD11b$^{-/low}$CD3-NK1.1+ cells gated as shown in figure 1 were analysed for the expression of CD49a and CD49b and the transcription factors that define their identity. A) Representative contour plots of the expression of CD49a and CD49b in the CD3-NK1.1+ population of the CNS, spleen and liver. At the right, the graph shows the mean proportion of each subset in each organ, n ≥ 5. B) Representative contour plots showing the expression of Eomes, Tbet and RORγt in the different CD3-NK1.1+ subsets of the CNS, at the right the graph shows the proportion of Tbet+Eomes+ cells in each subset, n = 5. C) Representative contour plots of the expression of RFP in the RORc-fm mouse in each of the CNS CD3-NK1.1+ subsets, the graph at the right shows the proportion of RFP+ cells in each subset, n = 3.

6.2.3 NK cells present in the CNS have a mature phenotype, ILC1s are CD11b^{low} and CD27+

To clarify the phenotype of the *bona fide* NK cells present in the CNS, the maturation markers were reassessed in the CD3-NK1.1+CD49a-CD49b cells (from now on referred as NK cells) and the CD3-NK1.1+CD49a+CD49b- cells (from now on referred as ILC1s). As shown in Fig. 10 A, the NK cells present in the CNS have a predominantly mature phenotype, with less than 15% of them constituting immature NK cells. In the case of ILC1s, cell subsets could not be classified as in the NK cells, but it was found that ILC1s have a low expression of CD11b and that most of them are CD27+. Furthermore, the expression of CXCR3 was reanalysed in the *bona fide* NK cells of the CNS. Still, a fraction of immature and mature NK cells expressed CXCR3, but it was not expressed in fully mature NK cells (Fig. 10B).

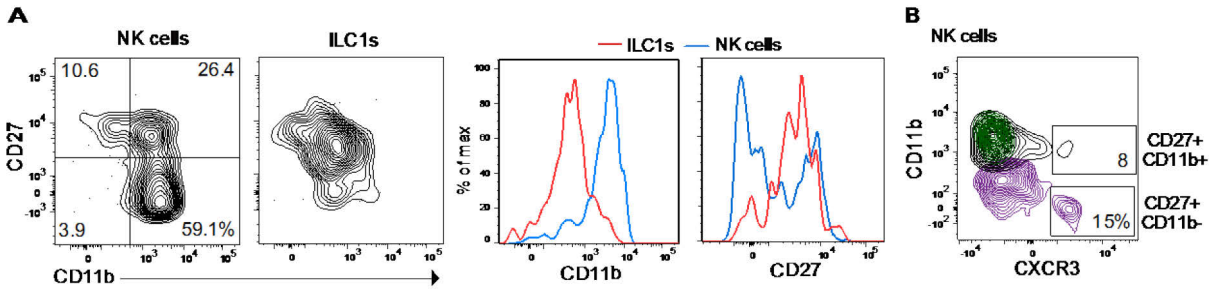

Figure 10. Maturation markers and CXCR3 in the group 1 ILCs of the CNS. A) Comparison of the expression of the markers CD27 and CD11b in the NK cells and ILC1s of the CNS, the numbers in the contour plot represent the proportion of each population in a concatenated sample of 3 mice. On the right side of panel A, a representative histogram overlay shows the fluorescence intensity of CD11b and CD27 in the NK cells and ILC1s. B) Contour plot overlaid of the NK cell maturation subsets showed in A, displaying the expression of CXCR3. The purple population represents the CD27+CD11b- subset, the gray the CD27+CD11b+ subset and the green the CD27-CD11b+ subset. Numbers represent the proportion of CXCR3+ cells.

6.2.4 ILC1s of the CNS are characterized by the expression of CXCR6, TRAIL and DNAM-1

After confirming that the NK1.1+ cells in the CNS are composed of different ILC populations, the ILC1 subset was further characterized in comparison with the NK cells. Therefore, the expression of diverse markers that are characteristic of ILC1s or NK cells were assessed. The intILCs were excluded from the analysis because of their reduced number and mixed identity. In addition, the 'ex-ILC3' that are part of the ILC1 population were included, as it has been shown that ILC3s and ex-ILC3s adapt their phenotype to the tissue microenvironment where they reside (87).

As expected, ILC1s expressed the tissue resident marker CD69 (79 ± 2 %), however they did not express the integrin CD103. Most notably, all ILC1s homogeneously expressed CXCR6 (97 ± 0.8 %), TRAIL (81 ± 6%) and DNAM-1 (96 ± 1 %). Furthermore, DNAM-1 expression was high in all the ILC1s and intermediate in the NK cells (DNAM-1 gMFI ILC1s = 24927; NK cells = 1760; p<0.0001). The distinctive marker of ILCs, the IL-7Rα was expressed in ILC1s in a heterogeneous manner (24 ± 2 %) while Thy1.2 expression was more frequently expressed in ILC1s (81 ± 4 %) as well as the recently proposed ILC1 marker CD200R (58 ± 5%). Interestingly, Perforin was also expressed in ILC1s (95 ± 1 %) but in a significant lower density than in the NK cells (perforin gMFI ILC1s = 2316; NK cells = 10561; p=0.006). Finally, c-Kit, a marker expressed in LTis and some ILC3 and ILC2 subsets, was not expressed in the CNS group 1 ILCs.

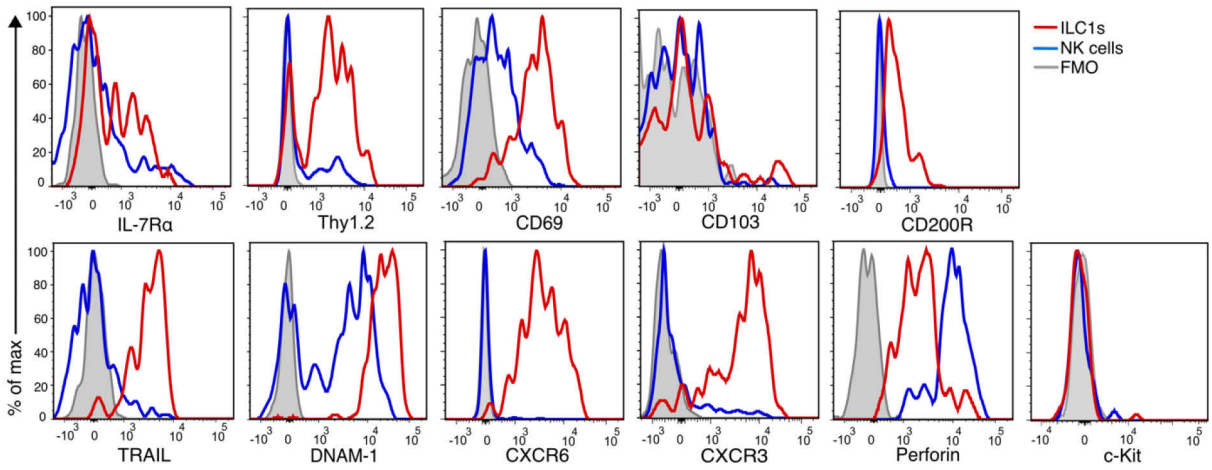

Figure 11. Phenotypic profile of group 1 ILCs of the CNS. Histogram overlays showing the expression of the indicated marker in the ILC1 and NK cell subset from the CNS. FMOs (from the CD3-NK1.1+ gate) are included as a reference for negative expression of the marker. Representative histograms of n ≥ 4 mice, from at least 2 independent experiments.

6.2.5 Besides CXCR3, ILC1s modulate TRAIL expression

After defining the phenotype of the CNS ILC1s, their response during neuroinflammation was next examined. Since it is known that inflammatory signals can modify the surface integrin expression in immune cells, it was first defined whether the surface expression of CD49a and CD49b were still a valid marker for the identification of group 1 ILC subsets during neuroinflammation. At peak EAE, the ILC1s, intILC1s and NK cells identified by the surface expression of CD49a and CD49b, expressed the expected transcription markers in the same way as in the naïve mouse. Interestingly, the inflammatory microenvironment had no effect in the proportion of intILC1s that expressed Eomes (Fig 12, left upper panel). These data indicate

that CD49a and CD49b are reliable markers to identify the different ILC group 1 subsets in a context of Th1/Th17 inflammatory environment, as is the case in the EAE.

Next, the stability of some of the defining ILC1 markers like DNAM-1, TRAIL, CD69 and IL-7Rα during neuroinflammation was explored. Most of the markers displayed the same expression pattern in the EAE sick mice as in the naive mice, except for CXCR3 (Fig. 12 right lower panel), which expression decreased in the ILC1s and NK cells as previously shown (Fig. 4). In addition, the percentage of ILC1s expressing TRAIL decreased during EAE (Fig. 12 middle lower panel). In addition, an increase of CD69 expression was also detected in the NK cells in the EAE mice (Fig 12 right upper panel).

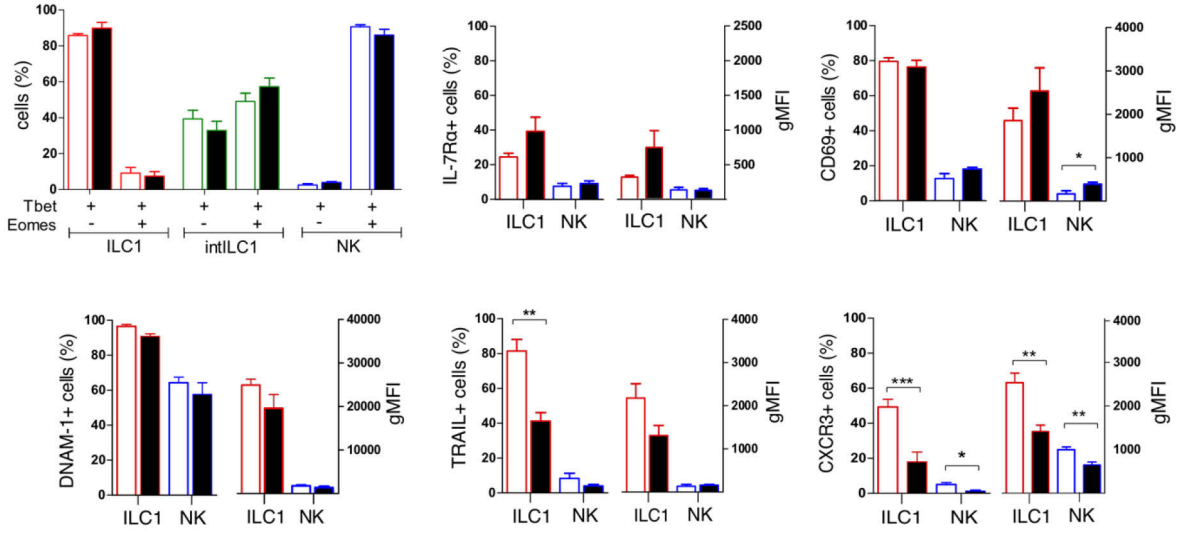

Figure 12. Transcription factor and surface marker expression during EAE. The expression of the indicated markers was compared between naïve (open bars) and EAE sick (black bars) mice in the ILCs and NK cells from CNS samples. N: transcription factors = 5 per group; surface markers ≥ 4 per group. Data from at least 2 independent experiments. The difference between the naïve and EAE group was analysed with a t-test *p<0.05, **p<0.01, ***p<0.001. gMFI, geometric mean fluorescence intensity.

6.2.6 ILC1s express more TNF-α than NK cells in the healthy and inflamed CNS

Next, the capacity of each subset to secrete the signature type I cytokines IFN-γ and TNF-α was explored. Cells isolated from naïve CNS were incubated with IL-15 and IL-12, which is known to induce IFN-γ secretion in both NK cells and ILC1s. Indeed, a high proportion of IFN-γ expressing cells was detected by flow cytometry after IL-15 and IL-12 stimulation, however, there was no production of TNF-α (Fig 13A). A more general stimulation protocol was performed using PMA and Ionomycin. This elicited also the production of IFN-γ. However, only ILC1s produced TNF-α. This was confirmed in cells derived from the CNS of sick EAE mice, that were restimulated with PMA and Ionomycin (Fig 13B). IFN-γ was produced by ILC1s

and NK cells in a comparable manner (as measured by the geometric mean fluorescence intensity (gMFI) of IFN-γ). However, a higher frequency of ILC1s expressed TNF-α and the expression was also more intense (Fig. 13B). Interestingly, the proportion of ILC1s producing both IFN-α and TNF-α was also higher in comparison with NK cells (Fig. 13B). Interestingly, the comparison of the cytokine production between the cells isolated from the naïve and EAE CNS revealed that NK cells, but not ILC1s, produce significantly more cytokines during EAE (Fig. 13C).

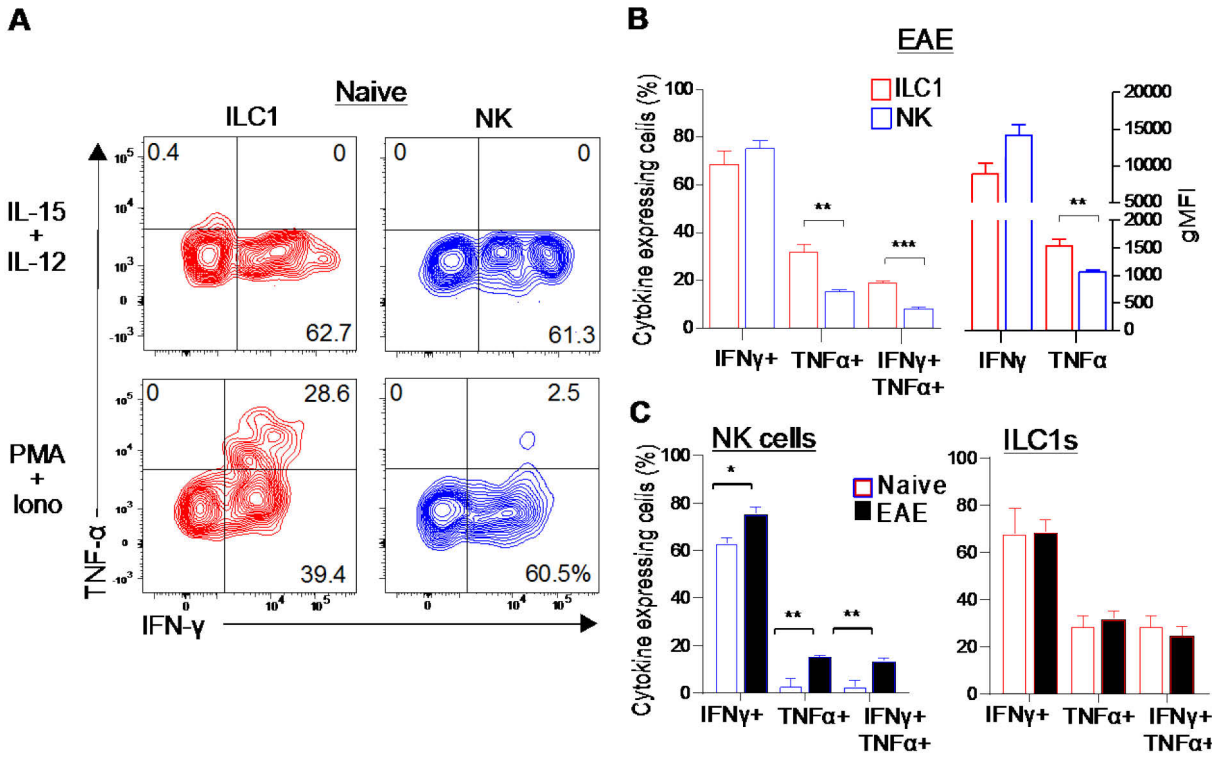

Figure 13. Cytokine production of group 1 ILCs of the CNS. Cells obtained from the CNS were stimulated for 5 hours in the presence of stimulants and Brefeldine A, stained for intracellular IFN-γ and TNF-α and analysed by flow cytometry. A) Representative contour plots showing the expression of IFN-γ and TNF-α in CNS from naïve mice after stimulation with IL-15 (50 ng/ml) and IL-12 (10 ng/ml) or PMA (50 ng/ml) and Ionomycin (Iono) (1µg/ml). The numbers are the mean percentage of 3 mice for each condition. B) Graph shows the mean + SEM of cytokine expression quantified as the percentage of expressing cells and gMFI in the ILC1 and NK cells of the CNS from sick EAE mice, stimulated with PMA and Ionomycin as in (A) N= 3, C) Percentage of cytokine expressing NK cells (left) and ILC1s (right) comparing between cells obtained from the CNS of naïve and EAE mice, N=3. Difference between groups was analysed with a t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.7 Group 1 ILCs are differentially distributed within the CNS compartments and are increased in the brain parenchyma during EAE

So far, the characterization of group 1 ILCs was performed in the whole CNS that include the brain with its choroid plexuses, the spinal cord and possibly traces of leptomeninges, specially pia matter. Since ILCs are known to be enriched in barrier structures, it was interesting to define whether the group 1 ILCs had a preferential location within the CNS compartments. Therefore, the choroid plexus was removed from the brain ventricles, and the dural meninges from the skull cap of the same mouse and the proportion and number of each subset was analyzed in the naïve and EAE condition. Interestingly, while in the naïve mouse, a dominance on the proportion of ILC1s was observed in all the compartments, during EAE, an overall increase of NK cells was observed, with a significant increase observed only in the brain parenchyma (Fig. 14 right panel). The choroid plexus was enriched with ILC1s and contained very low numbers of NK cells in the naïve mice (Fig. 14 middle panel). From the three compartments, the meninges contained the higher numbers of cells, that comprised all group 1 ILC subsets (Fig. 14 lower panel), as well as in the brain, where a significant increase of ILC1s and most notably of intILC1s and NK cells was observed during EAE (Fig 14 upper panel).

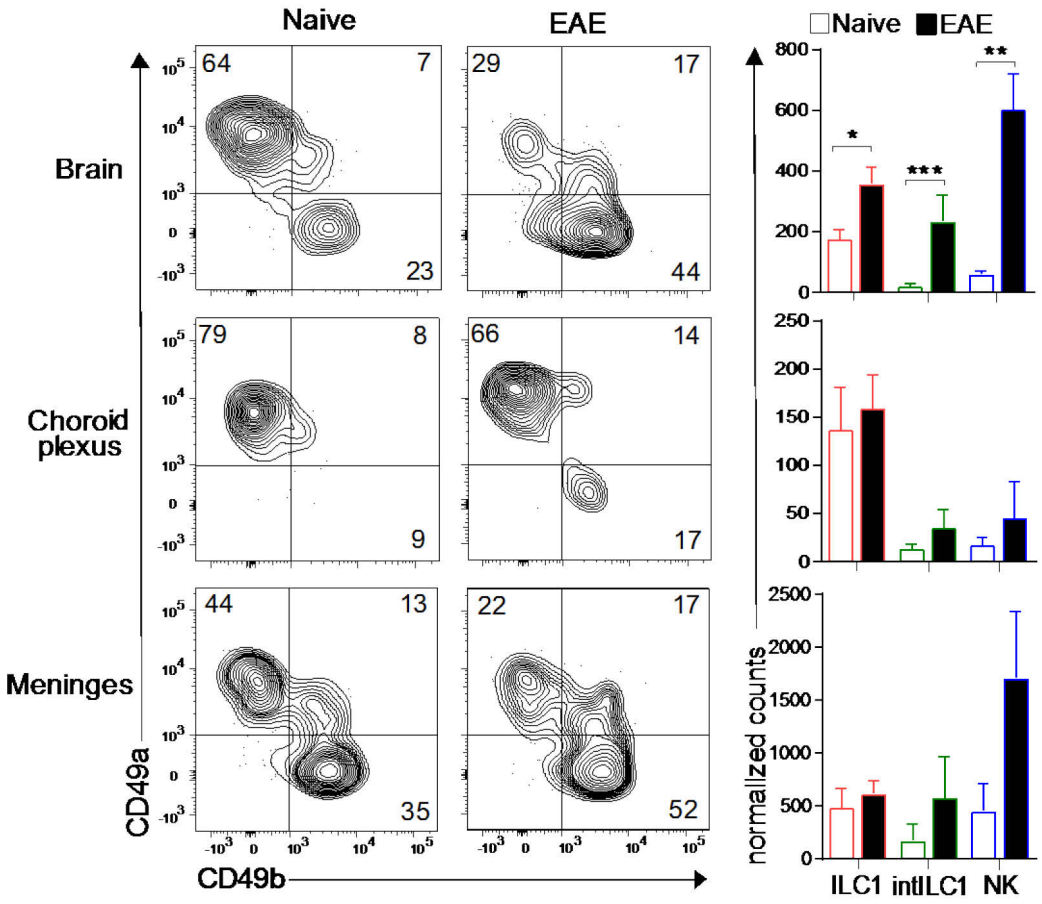

Figure 14. Distribution of group 1 ILCs within the CNS compartments. Representative contour plots showing the distribution of group 1 ILCs in the brain devoid of choroid plexus, the choroid plexus and the dural meninges. The numbers represent the mean percentage of each population. At the right, the graph shows the numbers of each subset normalized to live single cells. The graphs and numbers represent 6 mice per group in the brain compartment; 2 mice were pooled together in the case of choroid plexus and meninges in each condition, resulting in a n = 3. The naïve and EAE group were analysed with a t-test *p<0.05, **p<0.01. Graphs shown mean + SEM.

6.2.8 NK cells, but not ILC1s, proliferate in the CNS during EAE

The increase on NK cells in the brain during EAE is in line with our previous results of NK cell recruitment into the CNS during neuroinflammation (115). The increased numbers of group 1 ILCs on the brain parenchyma during EAE could also point to a proliferation of these subsets in response to neuroinflammation. Therefore, the marker Ki67 – a nuclear protein that is necessary for cell proliferation expressed during the active phases of the cell cycle (G1, S, G2, and M phases) – was used to assess the presence of proliferating cells by flow cytometry in the group 1 ILC subsets of the naïve and EAE mice. Due to the difficulty of performing intracellular staining with very low number of cells, like in the choroid plexus, the complete CNS was used. Similar to the results of the previous section, and increase in the proportion of NK cells and a decrease of ILC1s was found during EAE in comparison to the naïve mouse.

Furthermore, the numbers of NK cells but not of ILC1s were increased in the CNS during EAE. In concordance, the NK cells expressing Ki67 were higher in the EAE condition, both when identified by the surface markers or by the transcription factors (Fig 15B). The intILC1 showed a more variable increase in their numbers and expression of Ki67 during EAE that was not significant. As the Tbet+Eomes+ cells were the most proliferative during EAE, it was also analyzed whether within the intILCs, the Eomes+ subset proliferated more than the Eomes- subset. However, neither the proportion of Eomes+ intILC1s nor the proportion of Ki67+ cells in the Eomes+ intILC1s was increased during EAE (Fig. 15C). These data indicate that NK cells, but not ILC1s, proliferate readily in the inflamed CNS.

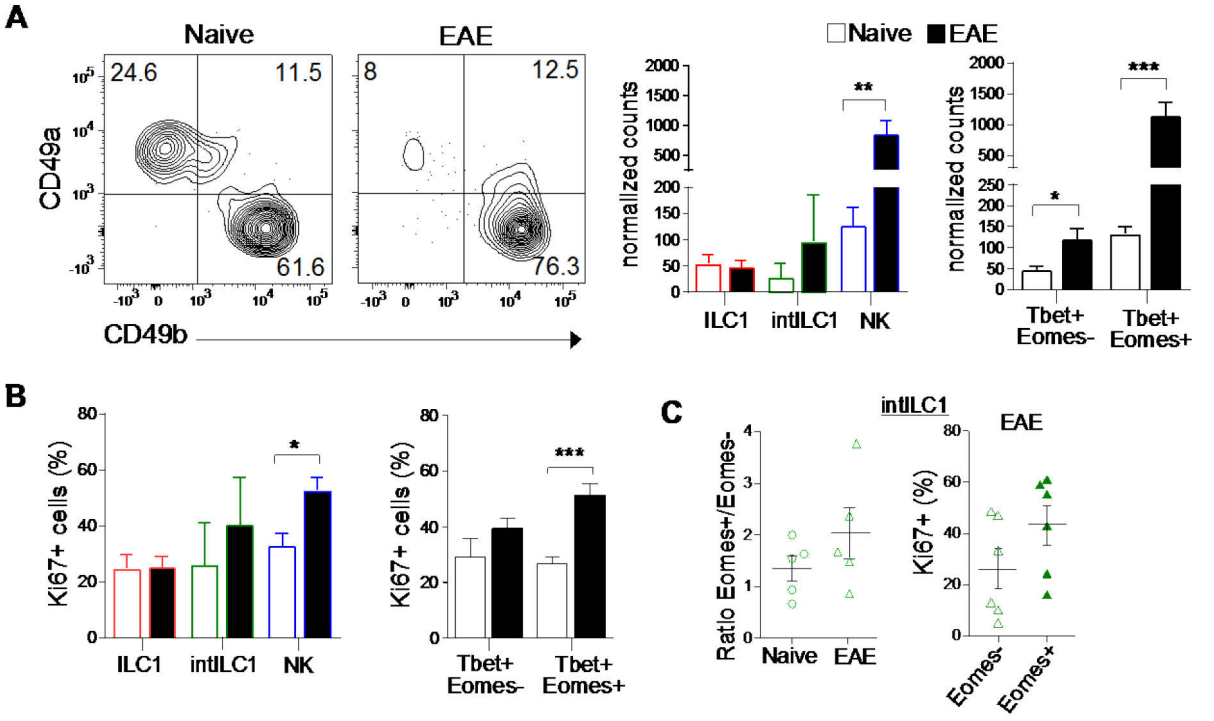

Figure 15. Proliferation of group 1 ILCs in the CNS during EAE. The numbers and proliferation of ILC1s, intILC1s and NK cells in the whole CNS were compared between the naïve and EAE mouse. A) Representative contour plot showing the proportion of each group 1 ILC subset in the CNS of naïve and EAE sick mouse. Numbers represent the mean proportion of each subset. The graphs at the right shows the comparison of the numbers of group 1 ILC subsets defined by the surface markers (left) or the transcription factors (right) in the naïve and EAE mouse as mean + SEM. N= 8 per group. B) Intranuclear expression of Ki67 was analysed by flow cytometry. The graphs show mean + SEM proportion of Ki67+ cells within each subset defined by the surface markers (left) or the transcription factors (right). N = 6 per group. C) The intILC1 subset was compared between the naïve and EAE condition in the proportion of Eomes+ cells represented as a ratio in the graph at the left. The frequency of Ki67+ cells was analysed in the Eomes- and Eomes+ intILC1s at the right. The line shows the mean ± SEM. A t-test was used to analyse the difference between the groups. *p<0.05, **p<0.01, ***p<0.001.

7 Discussion

NK cells, contrary to ILC1s, are able to circulate in the bloodstream, they home and reside in tissues like spleen and lung and participate in the immunosurveillance of the CNS (150). In the frame of the present thesis, two hypotheses were tested. First, assuming that the phenotypically immature NK cells present in the CNS originate from the periphery, the chemokine receptors that might contribute to the migration of immature NK cells into the CNS were investigated. Second, assuming that the cells identified as NK cells in the CNS are include an ILC1 fraction, an in-depth characterization of these cells with ILC defining markers was undertaken. The results obtained reveal a previously unrecognized diversity of group 1 ILCs present in the CNS of healthy mice and point to their differential response during neuroinflammation.

7.1 Characterization of the chemokine tools of immature NK cells in the CNS and periphery

The CNS is continuously surveilled by leukocytes coming from the periphery. Despite the restrictive nature of the CNS barriers, immune cells are found inside the CNS compartments, including the brain parenchyma, during steady state. This was recently demonstrated in a high dimensional analysis of the brain's immunological milieu using mass cytometry, in which the presence of DCs, neutrophils, monocytes, T cells, B cells and NK cells was determined (151). The immune cells present in the healthy CNS differ from circulating immune cells, which reflects a selective process at brain barriers like the CP that results in an enrichment of anti-inflammatory and immunomodulatory cells (218). This selection involves the interplay of chemokines, adhesion molecules, cytokines and their respective receptors expressed on the CNS barriers and immune cells. During neuroinflammation, an active recruitment and infiltration of immune cells into the CNS parenchyma occurs in a regulated manner, thanks to a switch to a pro-inflammatory microenvironment that loosens the restrictive nature of the barriers and facilitates cell infiltration (137), However, in advanced stages, a disrupted BBB can lead to a massive unregulated immune infiltration.

We previously showed that during EAE, mature NK cells are selectively recruited to the CNS in a CX3CR1-dependent manner. mature NK cells contributed to limit inflammation probably by lysing encephalytogenic T cells (115) (Figure 1). Furthermore, NK cells were present in CNS not only in EAE mice but also in healthy mice. Therefore, it was interesting to

determine the phenotype of NK cells that were present in the CNS during steady state and to determine the chemokine receptors that could mediate this recruitment.

It was found that the NK cells – defined as $CD45^{high}CD11b^{-/low}$CD3-NK1.1+ – that are present in the CNS of both healthy and EAE sick mice, include immature and mature NK cells with a predominance of immature CD27+CD11b- cells in the healthy mice (Figure 2). This finding was interesting since immature NK cells are enriched in the lymph nodes, while the NK cells present in the blood have a predominant mature phenotype. This suggests a similitude in NK cell recruitment mechanisms of the lymph nodes and the CNS. Furthermore, the presence of immature NK cells in CNS is in line with our previous report of an enrichment of $CD56^{bright}$ NK cells in the CSF of patients with inflammatory diseases (146).

Therefore, the expression of candidate chemokine receptors was evaluated to understand the mechanism of immature NK cell recruitment to the CNS during steady state. These candidates were CXCR3, CXCR4, CCR2 and CCR7, selected on the base of the known expression of the corresponding ligandsin the CNS, and cosindering the reports on CNS infiltration of immune cells that express these particular receptors as well as the known chemokine receptor expression of human and mice peripheral NK cells. Human $CD56^{bright}$ NK cells, for instance, express CCR7 (210). The endothelial cells of the BBB and the CP constitutively express CCL19, one of the CCR7 ligands, and the CSF is enriched with central memory T cells that express CCR7 and CXCR3 (145,218,219). Likewise, CXCL12, the ligand of CXCR4, is expressed in the abluminal surface of the BBB (138) and is involved in the retention of NK cells in the bone marrow (220). CCR2 is important for the recruitment of monocytes to the CNS during EAE (221). Finally, the expression of CXCR3 was reported in immature NK cells in various organs (48). In the present work, it was found that immature NK cells in the CNS express CXCR3 (about 30% of NK cells), and a small proportion CCR2 (less than 10%) (Figure 3,4). Because the expression of chemokine receptors is modulated in response to cytokines, the expression of these candidates was also evaluated during EAE. In this context, the expression of CCR2, CCR7 and CXCR4 on NK cells remained unchanged. However, CXCR3 expression dramatically decreased during neuroinflammation (Figure 4). This observation led to the investigation of the dynamics of CXCR3 expression on NK cells in *in vitro* experiments using NK cells isolated from lymph nodes and spleen.

Chemokine receptors are seven-transmembrane-spanning G-protein-coupled receptors whose activation is tightly regulated by different mechanisms including desensitization – a fast response that uncouples the receptor from the G protein – and internalization, in which the receptor is degraded or recycled, resulting in a more prolonged unresponsiveness to the ligand (222,223). The internalization of CXCR3 has been demonstrated in activated T cells and transfected cells lines (214,215,224). However, whether this is the case also in NK cells has

not been investigated. Here, using CXCL10, it was demonstrated that increasing concentrations of the CXCR3 ligand results in a disappearance of the receptor from the cell surface of NK cells isolated from LN (Figure 5). This result points to an internalization of CXCR3, since its downregulation was persistent after 4 hours of incubation with the ligand, a long time-frame for signalling dynamics. In addition, it is unlikely that the binding of CXCL10 could have hindered the anti-CXCR3 antibody binding to detect the receptor by flow cytometry because the cells were washed twice with cold PBS before performing the staining, which was also performed at 4°C, a non-optimal temperature for receptor-ligand binding kinetics. Although CXCR3 has three ligands, only the role of CXCL10 was tested because CXCL10 binds to the same epitope in CXCR3 as CXCL9, but to a different than CXCL11 (214). The role of CXCL11 was not explored because C57BL/6 mice are functionally deficient for CXCL11 due to a frame shift in the mRNA coding region that causes the truncation of the peptide (213).

Chemokine receptors, like other G-protein-coupled receptors, display biased signaling or functional selectivity. This means that certain situations can elicit the activation of one of several available signaling pathways. In the case of CXCR3, it has been shown that in murine activated T cells, the receptor activation with CXCL11 promotes a T cell polarization towards a Th2 or Tr1 subset, while the activation with CXCL9 or CXCL10 promotes a polarization towards a Th1 or Th17 subset. This biased signaling was dependent on STAT1, STAT4 and STAT5 in the case of CXCL9/10 activation of the receptor and on mTOR, STAT3 and STAT6 in the case of CXCL11 activation (217). The effect of CXCR3 signaling in the biology of NK cells has not been explored. Therefore, the effect of CXCL10 activation on the maturation and IFN-γ production on NK cells was evaluated in the present work. CXCR3 activation with CXCL10 did not affect the maturation status nor elicited IFN-γ production on isolated NK cells and sorted CXCR3+ NK cells derived from the spleen and LN after short term (4 hours) or long term (48 hours) incubation with the ligand (Figure 6). In these assays, NK cells were incubated with IL-15 to promote their survival, and their responsiveness was confirmed with the stimulation with IL-12 and IL-18 that are known to induce IFN-γ production and NK cell maturation (225). IL-12R signaling induces STAT4 phosphorylation that is critical for IFN-γ production (226). Therefore, these results suggest that CXCL10-induced CXCR3 signalling does not activate STAT4 on NK cells. Yet, effects of CXCL10 on other aspects of NK cell biology like cytotoxicity and proliferation were not explored in this study and cannot be ruled out. The observed reduction on the percentage of CXCR3+ NK cells found in the inflamed CNS may reflect an ongoing activation of the receptor by its ligands, which are upregulated in the CP, CSF, astrocytes and perivascular space during neuroinflammation (139,144,212,227); Further, the data indicates that this activation does not affect NK cell maturation and IFN-γ production.

Next, to explore if CXCR3 is involved in the recruitment of immature NK cells to the CNS during EAE, EAE mice were treated with a blocking CXCR3 antibody during the course of the disease. The results rather suggest, that CXCR3 might be involved in the homing of immature NK cells to the spleen, since a significant decreased proportion of immature NK cells and higher proportion of mature NK cells was found in that organ at day 15 post immunization in the treated mice compared to the IgG control mice (Figure 7). We previously reported a mobilization of NK cells in the periphery during the course of EAE. Interestingly, the decrease on the number of NK cells in the spleen correlates with an increase on the numbers of NK cells in the CNS at day 10 post immunization (115), which points to a mobilization of these cells from the spleen to the CNS. Furthermore, the immunization to induce EAE involves a systemic immune response to CFA and pertussis toxin i.p. injection protocol. In this context, IFN-γ production in response to the immunization protocol might increase the expression of the CXCR3 ligands in the spleen. Of note, although Th1 cells preferentially express CXCR3, the numbers and percentage of CD3+ T cells in the examined organs was not affected in the mice treated with the CXCR3 blocking antibody (not shown).

In a model of intracerebral infection of a murine coronavirus that expressed CXCL10, it was shown that NK cells were recruited to the CNS contributing to the clearance of infection. This NK cell recruitment of was detected as early as 2 days post infection (228). In our model, the brain was examined at day 15 post immunization, a time when the EAE disease should be already stablished (peak disease). The CXCR3 dependent recruitment of NK cells into the CNS in earlier stages of the EAE course, like the pre-onset and onset of disease deserves further investigation. In addition, the results of the here presented pilot experiment should be taken with caution due to its low power (n = 3 and n = 2 in the control-IgG and anti-CXCR3 group, respectively).

In sum, these results indicate that immature NK cells express CXCR3, which is actively internalized after the engagement with its ligand. However, CXCR3 did not seem to mediate the recruitment of immature NK cells into the CNS during inflammation, but rather contribute to their homing to the spleen in the EAE model. It is possible that other chemokine receptors unexplored in the present study might be involved in the specific migration of immature NK cells into the inflamed CNS in the context of EAE.

7.2 Identification of different ILC group 1 subsets in the CNS

If the immature NK cells present in the CNS during steady state and inflammation are not coming from the periphery, an alternative is that they represent tissue resident cells. ILCs are tissue resident cells that have been described in various organs. Due to the phenotypical similitudes between NK cells and ILC1s, it was crucial to investigate the CD3-NK1.1+ cells of the CNS with more detail to elucidate their true identity and generate appropriate models and research questions. The second part of the present study was dedicated to this task, which generated new insights into the ILC composition of the healthy CNS and the specific phenotype and functionality of the identified ILC1s in comparison to the conventional NK cells.

The expression of CD27 and CXCR3 on the immature NK cells suggested a possible ILC1 identity (17,96). Therefore, the expression of the integrins CD49a and CD49b was also investigated to roughly differentiate between ILC1s and NK cells, respectively, within the maturation subsets of the CD3-NK1.1+ cells of the CNS. This analysis revealed that immature CD3-NK1.1+CD27+CD11b- cells co-expressed CD49a (about 80% of cells) in a higher proportion than CD49b (about 20% of cells). Conversely, the mature (CD3-NK1.1+CD27+CD11b+) and fully mature (CD3-NK1.1+CD27-CD11b+) NK cells co-expressed CD49b in high proportion (about 80% of cells), while few or no cells co-expressed CD49a in the mature (about 20%) and the fully mature population (less than 5%) (Figure 8). These data indicated that a fraction of immature NK cells display a ILC1 phenotype.

To further confirm the presence of ILC1s, the expression of the same integrin markers was evaluated directly in the CD3-NK1.1+ cells. Three populations were identified, a CD49a+, a CD49b+ and a double positive population. The transcription factor analysis revealed that these populations are Tbet+, but that differentially express Eomes. Eomes expression is necessary for the development, maintenance and function of NK cells (25–27). In this line, the CD3-NK1.1+CD49b+ expressed Eomes, confirming their identity as conventional NK cells, while CD3-NK1.1+CD49a+ were Eomes- indicating a ILC1 identity. Intriguingly, 50% of the CD49a+CD49b+ population expressed Eomes+, and was thus termed intILC1s (Figure 9B). It is possible that the presently described Eomes+ intILC1s are NK cells with an upregulated expression of CD49a and that the Eomes- intILC1s are ILC1s with an upregulated expression of CD49b. However, this population has been described in other contexts. On one hand, Gao and colleagues found a high proportion of tumor associated CD49a+CD49b+ intILC1s. They showed by global transcriptome analysis that intILC1s have a different gene expression profile than ILC1s and NK cells, including a low Eomes expression, proposing the intILC1s as an independent subset (68). On the other hand, Filipovic and colleagues showed in the uterine group 1 ILCs, that a proportion of NK1.1+CD49a+ cells were Eomes+, which they called tissue

resident NK (trNK) cells. In this case, transcriptome analysis showed a closer relationship in the gene expression profile between trNK cell and ILC1s, than cNK cells and ILC1s. Interestingly, uterine ILC1s clustered away from liver ILC1s revealing the uniqueness of the uterine ILC1s (101). Furthermore, salivary gland ILC1s are CD49a+CD49b+ and express both Tbet and Eomes. However, it was shown that these cells are not dependent on either transcription factor. In addition, transcriptome analysis revealed a unique gene expression profile of the intILC1s that shared some characteristics with ILC1s and NK cells (92). Similarly, a transcriptome analysis would be instrumental in our studies to define the relationship of the presently described intILC1s in relationship with the ILC1s and NK cells, as well as to ascertain the similitude of the CNS-ILC1s with the ILC1s of other organs like the liver.

A subset of group 3 ILCs express NK1.1 and NKp46. It was confirmed that ILC3s were absent from the here defined CNS group 1 ILCs as none of them expressed RORγt, the defining transcription factor of ILC3s. However, ILC3s are able to convert into ILC1s (65,229). Therefore, it was also investigated if some of the presently described ILC1s had an ILC3 origin by using the RORc-fate mapping mouse that permanently marks cells that expressed RORγt at some point during their ontogeny. In this way, it was revealed that about 35% of the ILC1s and intILC1s have an ILC3 origin (Figure 9C). Ex-ILC3s were also found in a similar proportion in the liver (33 ± 5 %) of the same mice (data not shown). Thus, it would be interesting to determine in future experiments the baseline proportion of ex-ILC3s in the different tissues and how that changes in response to inflammatory challenges. Importantly, a differential functionality of ex-ILC3s has been reported. In the gut, ex-ILC3s were found to exacerbate experimental colitis (93). RORc^{fm+} ILCs (which include exILC3s and ILC3s) were found to have more potent anti-tumoral properties than RORc^{fm-} ILCs in the spleen (87). Therefore, it is of great interest to address in future experiments the differences between the CNS ILC1s and ex-ILC3s on a functional level.

On the other hand, it was shown in adoptive transfer experiments that the phenotype of RORc^{fm+} ILCs is shaped by the tissue microenvironment. For example, transferred RORc^{fm+} ILCs isolated from the spleen homed to different organs, when recovered from the host organ they no longer displayed a spleen-ILC phenotype but an ILC phenotype characteristic of the organ, like a low Thy1.2 expression if recovered from the liver, or a high Thy1.2 expression if recovered from the small intestine lamina propia (87). These data indicate that the phenotype of RORc^{fm+} ILCs, and probably of ILCs in general, is imprinted by the tissue microenvironment where they reside. Thus, the elucidation of the specific phenotype of the ILCs that reside in such a unique organ as the CNS is of great interest.

Once the group 1 ILC subsets of the CNS were defined, the re-assessment of the maturation stages of *bona fide* NK cells present in the CNS was imperative. It was found that

CD3-NK1.1+CD49b+ comprise mostly mature and fully mature NK cells with a small fraction of immature cells (10% of NK cells) (Figure 10A), these immature NK cells expressed CXCR3 in a lower proportion than previously defined (15% vs 60% of immature NK cells, figs. 10B and 3C, respectively). The ILC1s could not be classified by the classical NK cell maturation markers, but they expressed high levels of CD27 and low levels of CD11b. These results might explain why the systemic CXCR3 blocking treatment did not affect the proportion of NK cells in the CNS (Figure 7), as most of the CXCR3+ cells are actually ILC1s (Figure 4) and only a small fraction of immature NK cells express this chemokine receptor.

The phenotype of the type ILC1s was further characterized in comparison with the NK cells of the CNS (Fig 11). Moreover, the modulation of some of the found markers was further evaluated in EAE mice (Fig. 12). Although CD127 (IL-7Ra) is a defining marker of helper ILCs, it was not expressed in all CNS ILC1s but only in a fraction (24 ± 2 %) (Fig. 11). A small proportion of NK cells also expressed CD127 (7 ± 1 %). Similarly, it has been reported that liver, skin, uterus and salivary gland ILC1s have a moderate or no expression of CD127 (96,98). It was shown that contrary to ILC2 and ILC3, ILC1s are not affected by IL-7Ra deficiency, but that like NK cells, depend on IL-15 for their development and maintenance (17).

CD90.2 (Thy1.2) was also expressed in ILC1s in high proportion (81 ± 4 %) (Fig. 11). This marker is expressed consistently in helper ILCs (7), however it is not an ILC exclusive marker since it is also expressed in T cells, hematopoietic stem cells and other non-lymphoid cells such as neurons (230,231). In this line, a low proportion of NK cells also expressed CD90.2 (39 ± 4%).

CD69 is a tissue resident marker, it interacts with S1P1 inhibiting the response to the S1P gradient present in the bloodstream (123). In concordance, ILC1s homogeneously expressed CD69 (79 ± 2 %) while some NK cells expressed it in lower levels (12 ± 2 %) (Fig. 11). CD69 expression significantly increased on NK cells in the EAE mice (Fig. 12). CD69 is also considered as an early activation marker. Its upregulation is therefore in line with an activation of NK cells in the EAE microinflammatory environment. CD69 upregulation on immune cells after activation contribute to their retention in the site of inflammation. CD103, an integrin that is expressed in intraepithelial ILC1s (60) was not expressed in the CNS-ILC1s.

CD200R is an anti-inflammatory receptor expressed in myeloid cells, its expression has been found also in T and B cells (232), and only recently in ILC1s but not NK cells of the peritoneal cavity (62). Here, an expression of CD200R was also found in CNS-ILC1s (58 ± 5%) but not NK cells (Fig. 11). In the CNS, CD200R is expressed in microglia and astrocytes and the ligand, CD200, is expressed in endothelial cells and neurons (233). The CD200R-CD200 axis appears to be an immunomodulatory mechanism by which neurons suppress microglia activation. CD200 deficiency as well as the blockage of CD200R-CD200 interaction

result in a more severe EAE disease, (234–237). Conversely, the administration of a CD200R agonist attenuated EAE disease (238). The presence of CD200R in CNS-ILC1s might be therefore a mechanism to inhibit undesired immune responses of CNS resident ILC1s against neurons and support a homeostatic/immunomodulatory role of these cells.

In general, ILC1s are considered to be non-cytotoxic, however, it was found that CNS-ILC1s express perforin (95 ± 1 %), albeit in lower levels than NK cells (perforin gMFI ILC1s = 2316; NK cells = 10561; p=0.006). In addition, TRAIL is an apoptosis inducing ligand that was also found to be homogeneously expressed on the CNS-ILC1s (81 ± 6%), and on few NK cells (8 ± 2 %) (Fig. 11). The expression of TRAIL is part of the ILC1 signature (239), low levels of perforin in ILC1s have also been reported. Tonsil intraepithelial ILC1s express perforin and are able to release lytic granules in cytotoxicity assays with tumor cell lines (60). Liver ILC1s are also perforin^{low} (56,240) and express TRAIL and are able, like salivary gland ILC1s, to mediate target cell killing via TRAIL (55,61,98). It was shown that neurons upregulate TRAIL-R2 during the course of EAE, which make them vulnerable to TRAIL mediated killing by encephalitogenic T cells. The authors showed that a brain specific blockade of TRAIL reduced EAE severity as well as the transfer of TRAIL-deficient T cells (241). On the other hand, mice systemically treated with a blocking soluble TRAIL receptor (242) and TRAIL-deficient mice display a more severe EAE disease (243). In the TRAIL-deficient mice, a greater frequency of CD4+ Th1 cells, and a lower frequency of CD4+Foxp3+ Tregs was found compared to WT mice (243). In this line, various reports have shown that TRAIL is important to inhibit T cell activity by Tregs and NK cells, either by killing them or inhibiting their proliferation (244–247). In the same line, it was found that CNS-ILC1s homogeneously express DNAM-1 (96 ± 1 %) in much higher levels than NK cells (DNAM-1 gMFI ILC1s = 24927; NK cells = 1760; p<0.0001) (Fig. 11). DNAM-1 is another activating receptor that has been implicated in the killing of autoreactive T cells by NK cells (193). Of note, MS patients displayed lower levels of DNAM-1 in NK cells and its ligand CD155 on activated T cells. Daclizumab treatment restored the expression of these proteins facilitating NK cell cytolitic activity against activated T cells in MS patients (193). Here, it was found that the expression of DNAM-1 was stable in the CNS-ILC1s during EAE, however, a downregulation of TRAIL on ILC1s was detected on EAE mice (Fig. 12). TRAIL can be released in a soluble form from granules (248) or after being cleaved from the surface by cysteine proteases (249). In addition, it was shown that TRAIL death receptors are endocytosed together with bound TRAIL to regulate the apoptotic signaling (250). Thus, the downregulation of TRAIL on ILC1s might reflect either a cleavage to generate soluble TRAIL, or an internalization after being bound to the death receptors in target cells.

In sum, the characteristic high expression of TRAIL and DNAM-1 in CNS-ILC1s suggest that they might be able to recognize and lyse activated T cells.

The chemokine receptor CXCR6 was also expressed homogeneously in all CNS-ILC1s (97 ± 0.8 %) but not on NK cells (Fig. 11). CXCR6 expression has been detected in the ILC1s of the liver, salivary glands and uterus (60,92,101,239) In the CNS, its ligand, CXCL16 is expressed in endothelial cells, astrocytes, microglia, and neurons (251,252) and its expression has been shown to be upregulated in vitro by IFN-γ and TNF-α. Furthermore, astrocytes release soluble CXCL16 upon inflammatory conditions (251) and an elevated concentration of CXCL16 has been found in the CSF of MS patients (253). Interestingly, the CXCL16-CXCR6 axis promote neuroprotection in a model of glutamate exitotoxicity and cerebral ischemia (252,254). On the other hand, CXCR6+ NK1.1+ cells have been assigned a memory function in the liver and uterus (101,117). Liver memory NK cells – defined as CD45+NK1.1+Thy.1+, most likely representing ILC1s – mediate hapten-induced contact hypersensitivity responses, that is, they mount a memory response in the skin against haptens to which mice have previously been sensitized (255). Paust and colleagues showed that these memory NK cells are CXCR6+, that CXCR6 blockade abolish memory responses and that CXCR6-deficient NK cells are not able to transfer hapten sensitivity and survive poorly. CXCR6 did not mediate the antigen recognition but rather seemed to mediate memory NK cell homeostasis and survival through the engagement with CXCL16 that is constitutively expressed in hepatic sinusoidal endothelium (117). In the uterus, CXCR6+ ILC1s expanded in second pregnancies suggesting a pregnancy memory role of these cells, which are probably responding to the CXCL16 expression by the trophoblast (101). Of note, the expression of DNAM-1 was also found to be essential for the differentiation of memory NK cells in response to MCMV infection (53). In sum, considering the expression of CXCL16 in the brain parenchyma, it is likely that CNS-ILC1s reside in close proximity with the brain cellular components where they can mediate neuroprotective and/or memory functions. Therefore, it would be of great interest to study the response of CNS-ILC1s to EAE relapses in the relapsing-remitting SJL model. In addition, it is likely that CXCR6 signaling is important to promote the survival and maintenance of CNS-ILC1s, as it was shown in the liver ILC1s (117).

Moreover, it was confirmed that CXCR3 is predominantly expressed on ILC1s (49 ± 4 %) and that its expression is decreased on both NK cells and ILC1s during EAE disease (Fig. 12). As shown in the experiments of the previous section, the internalization of CXCR3 seen on LN- and spleen-derived NK cells after encounter with CXCL10 (Fig. 5), should also apply to ILC1s. Hence, CXCR3 downregulation indicate that ILC1s might reside in locations where CXCL10 ligands are expressed during inflammation such as the choroid plexus (144) and the subventricular zone (SVZ), in which NK1.1+ cells have been found to accumulate at chronic stages of EAE (206). In that context, it was shown that SVZ neural stem cells (NSCs) were a source of IL-15 to sustain NK cells, however, a backlash effect of NK cell cytotoxicity against NSCs hindered recovery from chronic EAE. NSC were shown to downregulate the inhibitory

ligand Qa1 in chronic EAE, which may have made them more susceptible to killing by NK cells (206). Whether ILC1s express the same array of inhibitory receptors as NK cells, such as NKG2A that recognizes Qa1, is unclear, and how they respond to missing self signals is a matter of further investigation.

In sum, the phenotypic profiling of CNS group 1 ILCs show that CXCR6, TRAIL, DNAM-1^{high} and CD200R are defining markers of ILC1s in this tissue, which hints into a homeostatic and immunomodulatory role of this subset in the CNS.

The expression of integrins and other adhesion molecules on the surface of the cells is highly dynamic and might change upon stimulation. Here, it was confirmed that the distinction of the group 1 ILC subsets of the CNS by the surface expression of CD49a and CD49b was still valid in the context of neuroinflammation. The expression of nuclear Tbet and absence of Eomes was unchanged in the CD49a+CD49b- ILC1 population in EAE mice. Likewise, CD49a-CD49b+ NK cells from EAE mice expressed nuclear Tbet and Eomes in the same way as naïve mice. In the CD49a+CD49b+ intILC1s, a slight but non-significant increase of Tbet+Eomes+ cells was observed in the EAE mice (Fig. 12).

It has been reported that ILC1s express a broader range of cytokines upon stimulation than NK cells, including IL-2, GM-CSF and TNF-α (56,61,96). Here, the expression of TNF-α and IFN-γ by the group 1 ILCs of the CNS was explored *in vitro* using cells derived from the CNS of naïve mice and EAE mice. When cells were stimulated with IL-15 and IL-12, a high proportion of NK cells and ILC1s expressed IFN-γ, but almost no TNF-α was detected. PMA and Ionomycin stimulation, in turn, elicited IFN-γ and TNF-α production. This was also the case in cells derived from the CNS of EAE sick mice. In concordance with previous reports, it was found that CNS-ILC1s produce significantly more TNF-α than NK cells, but they produced IFN-γ in a similar way as NK cells. In addition, significantly more ILC1s were able to produce both TNF-α and IFN-γ at the same time (Fig. 13B). Altogether, the phenotypic and cytokine secretion profile substantiate a true ILC1 identity of the NK1.1+CD49a+ cells of the CNS. Importantly, it was found that the expression of cytokines in ILC1s did not increase in EAE sick mice compared to the naïve mice (Fig 13C). Since ILC1s provide an early source of cytokines during inflammation (62), an increase in ILC1 cytokine production might have occurred at onset or pre-onset stages of EAE.

Next, it was interesting to determine the location of these group 1 ILCs within the CNS compartments. Their chemokine receptor profile supported a possible location in the brain parenchyma and the choroid plexus, indeed, ILC1s were found in those compartments and represented the most frequent population of the CD3-NK1.1+ cells. Interestingly, in the steady state, ILC1s were highly overrepresented in the choroid plexus (about 80% of NK1.1+ cells), while very few intILC1s and NK cells were found there. The ILC1s residing in the CP could

have a gatekeeper role, as it was previously shown for other IFN-γ secreting cells located in the CP. Specifically, it was shown that Th1 cells residing in the CP regulated the expression of trafficking molecules via IFN-γ secretion, which allowed the infiltration of immune cells during inflammation. Importantly, this regulation was shown to be favorable and necessary for the recovery from spinal cord injury by allowing the infiltration of beneficial monocytes (144). Furthermore, a shift towards a Th2 microenvironment in the CP was found in aged mice and was associated with cognitive decline (143). Similarly, CP-resident ILC1s might contribute to the beneficial type I IFN milieu that coordinates the selective trafficking of immune cells into the CSF. A gatekeeper function was also ascribed to ILCs present in the meninges, in one of the few reports that have addressed the contribution of ILCs to EAE pathology (207). In that study, Kwong and colleagues visualized numerous NKp46+ cells in the meninges of NKp46-reporter EAE mice, and showed that they were present there even before disease onset. In addition, mice with a specific deletion of Tbet in NKp46+ cells ($Tbx21^{f/f}$ NKp46-Cre+), which caused a reduction of ILC1s and NK cells but not NCR+ ILC3s, displayed a delayed EAE onset after Th17 adoptive cell transfer, indicating a role of NKp46+ ILCs in controlling infiltration of Th17 cells into the CNS. Less CD4 cell infiltration in the parenchyma and less expression of metalloproteinases and chemokines in the meninges was found in those mice, indicating that NKp46+ ILCs in the meninges express factors that facilitate cell infiltration during inflammation. The effect pointed to the ILC1s, since NK cell-deficient mice ($Eomes^{f/f}$NKp46-Cre+) did not display a delayed onset of Th17 adoptive cell transfer EAE (207). Here, it was found in concordance that the meninges contain the three group 1 ILC1 subsets, with a similar proportion of ILC1s (44%) and NK cells (35%). Interestingly, a significant increase on the numbers of all three group 1 ILCs was found only in the brain parenchyma in the EAE mice, with a notable increase in the numbers of intILC1s ($p<0.001$) and NK cells ($p<0.01$), which is in line with a recruitment of these cells into the brain parenchyma, where the myelin-targeted inflammation occurs. Besides an active cell recruitment from the CNS compartments into the parenchyma, an *in situ* cell proliferation in response to inflammation could explain the increased numbers of group 1 ILCs during EAE. Therefore, the cells that were actively proliferating were identified with the Ki67 marker in the whole CNS (Fig. 15). That analysis revealed that only NK cells had an increased proliferation in the CNS of EAE mice compared to naïve mice. Since a fraction of intILC1s also are Eomes+, the proliferation on this subset was also analyzed, however, there was not a significant increase in the number of Eomes+ intILC1s nor a higher percentage of Ki67+ cells on the Eomes+ intILC1s. In concordance with the previous result of an increase of ILC1s and intILC1s on the brain parenchyma during EAE, an increase on the number of Tbet+Eomes- cells was still observed when analyzing the whole CNS. It is possible that a dilution effect of having examined the whole CNS instead of each compartment separately could account for the lack of increased Ki67+ cells in the ILC1s and

intILC1s. However, it is also possible that the increased numbers of intILC1s and ILC1s in the brain parenchyma are caused by a conversion of NK cells into intILC1s/ILC1s under the inflammatory microenvironment of the EAE brain. It has been shown with *in vitro* and *in vivo* experiments that TGF-β can induce the differentiation of NK cells into ILC1s cells (68,92,256,257). TGF-βs are multifunctional cytokines that play a regulatory role in the initiation and resolution of immune responses. TGF-β1 is present in normal human brain tissue, astrocytes and microglia and has been found in active demyelinating lesions of MS patients (258). In the EAE model, TGF-β1 secretion by astrocytes and microglia is upregulated at early stages of disease (259,260). Therefore, it is likely that some infiltrating NK cells respond to it by converting into ILC1s via an intILC1s stage, which might explain the great increase on intILC1 numbers in the brain during inflammation.

In conclusion, the results of the present thesis provide new evidence of the existence of diverse group 1 ILCs in the healthy CNS of mice. The phenotypical characterization of CNS-ILC1s strongly suggest that they take active part as immunomodulatory, neuroprotective and gatekeeping agents in the CNS parenchyma and barriers. Finally, this work offers a foundation for further investigation of the implication of ILCs on CNS immunity and homeostasis.

7.3 Graphical summary

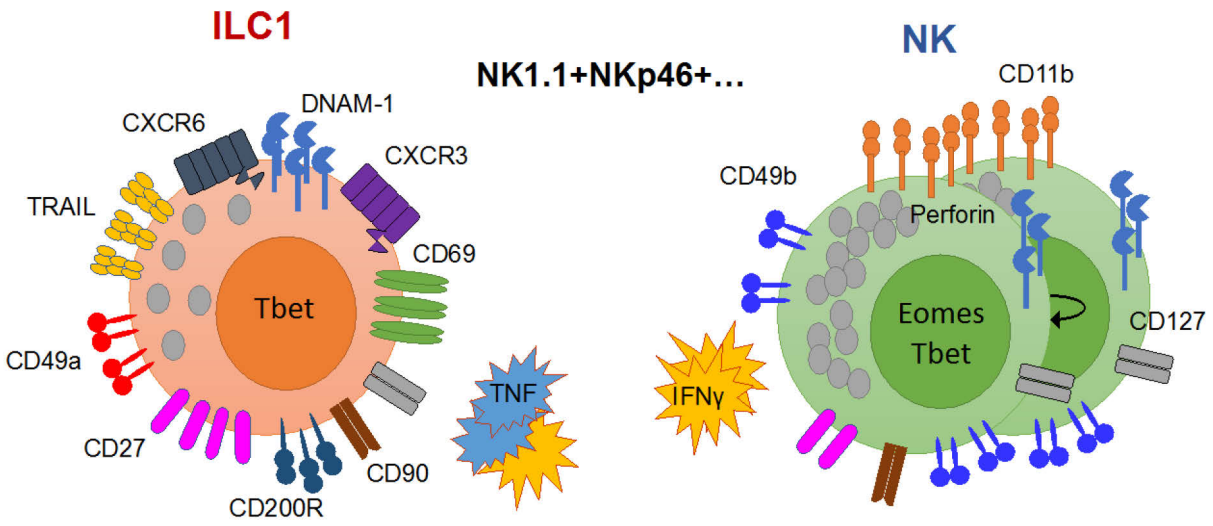

Figure 16. Phenotype of CNS-ILC1s and NK cells. ILC1s in the CNS express the transcription factor Tbet, have few intracellular perforin and secrete TNF-α and IFN-γ. They are characterized by the surface expression of CXCR6, CXCR3, DNAM-1, TRAIL, CD200R, CD49a, CD69 and CD27. NK cells express Eomes in addition to Tbet, have high levels of intracellular perforin and secrete predominantly IFN-γ. In overlap with ILC1s, they express lower levels of DNAM-1 and some express CD127 and CD90. They can have an immature phenotype characterized by CD27 but most of them express the maturation marker CD11b and express the integrin CD49b. In addition, they show a higher proliferative response than ILC1s in response to neuroinflammation.

7.4 Outlook

The long-term tissue residency and lack of recirculation of ILCs in diverse organs has been demonstrated multiple times with parabiosis experiments (61,62,67,92,96,105,261). Hence, in the present work, it is assumed that the newly described ILC1s of the CNS are tissue resident, a possibility that is substantiated by their expression of the tissue resident markers CD49a and CD69. However, that remains to be confirmed with parabiosis experiments. Of special interest would be the comparison of the recirculation dynamics of ILC1s with the NK cells, which are assumed to originate from the periphery but whose mobilization and retention in the CNS might differ from peripheral organs due to the immunoprivileged nature of the CNS. Recently, it was reported that hapten sensitization induced the mobilization of memory-ILC1s to the skin-draining LNs in a CXCR3-dependent manner. Memory-ILC1s were then recruited to the liver in a CXCR6-dependent manner (262). These findings challenge the view of an obligate tissue residency of ILCs and highlight the need to study the mobilization of ILCs during inflammation. Thus, the mobilization of the presently described CXCR3+CXCR6+ ILC1s between the CNS and the CNS-draining lymph nodes deserves consideration.

The elucidation of factors that maintain and support ILC1 functions in the CNS are of great relevance. Microglia display a TGF-β-dependent gene expression signature and they are absent in TGF-β1-deficient mice (263). Similarly, the maintenance of ILC1s in the CNS might be dependent on the TGF-β1-rich environment of the CNS, which is in concordance with reports of salivary gland ILC1s (92). In addition, it has been shown that NSCs are able to secrete IL-15, and that IL-15 is increased in the brain in conditions like enriched environment (206,264), further supporting a CNS intrinsic mechanism for ILC1 maintenance. In the same line, important questions to address are the pinpointing of the appearance of ILC1s or ILC progenitors in the tissue during CNS development, and whether CNS-ILC1s are replenished in the tissue by the conversion of NK cells into ILC1s.

Based on the phenotypic profile of the ILC1s described in the present thesis, the proposed roles of CNS ILC1s are a gatekeeping function in the brain barriers such as the CP and meninges and a neuroprotective role in the brain parenchyma. The role of ILC1s in the development of EAE was not explored in the present thesis. ILC1s could have a detrimental role on EAE by allowing the passage of autoreactive T cells as reported by Kwong (207), conversely, a protective role by limiting inflammation and lysing autoreactive T cells is also possible. Further studies with a ILC1-deficient mouse, such as the Hobbit knock-out mice, would shed light into the definite role of ILC1 in the onset and development of EAE. In addition, it is of great interest to study the response of ILC1s in pre-onset and chronic stages of EAE, since ILC1s were found to act early in inflammation by being the first source of IFN-γ in the

context of viral infection (62), but have also been associated with delayed, long-term memory responses (262).

In addition, since ILC1s orchestrate type I responses, it is of key importance to investigate the role of CNS-ILC1s in the clearance of viral infections, such as meningitis; and the control of brain tumors, such as glioma.

To conclude, the strategic location of ILC1s in the CNS open new avenues in the investigation of the cross talk between the immune system and the nervous system. Recently recognized is the fact that ILCs are able to respond to or produce neuropeptides, neurotransmitters, neurotrophic factors and hormones. For example, group 1 ILCs express the glucocorticoid receptor whose activation has been shown to inhibit IFN-γ production (265). In addition, it has been reported that NK cells are able to produce acetylcholine (266). Therefore, further research should capture details of the communication between group 1 ILCs, glia and neurons.

8 References

1. Melorose J, Perroy R, Careas S. *Immunobiology of Janeway*. (2015). doi:10.1017/CBO9781107415324.004

2. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KS, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature* (2015) **523**:337–41. doi:10.1038/nature14432

3. Parker GA, Picut CA. Immune Functioning in Non lymphoid Organs: The Liver. *Toxicol Pathol* (2012) **40**:237–247. doi:10.1177/0192623311428475

4. Jenne CN, Kubes P. Immune surveillance by the liver. *Nat Immunol* (2013) **14**:996–1006. doi:10.1038/ni.2691

5. Odoardi F, Sie C, Streyl K, Ulaganathan VK, Schläger C, Lodygin D, Heckelsmiller K, Nietfeld W, Ellwart J, Klinkert WEF, et al. T cells become licensed in the lung to enter the central nervous system. *Nature* (2012) **488**:675–679. doi:10.1038/nature11337

6. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, Walzer T. The trafficking of natural killer cells. *Immunol Rev* (2007) **220**:169–182. doi:IMR563 [pii]\n10.1111/j.1600-065X.2007.00563.x

7. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie ANJ, Mebius RE, et al. Innate Lymphoid Cells: 10 Years On. *Cell* (2018) **174**:1054–1066. doi:10.1016/j.cell.2018.07.017

8. Kiessling R, Klein E, Wigzell H. „Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* (1975) **5**:112–117. doi:10.1002/eji.1830050208

9. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* (1975) **16**:216–229. doi:10.1002/ijc.2910160204

10. Mebius RE, Rennert P, Weissman IL. Developing Lymph Nodes Collect CD4+CD3− LTβ+ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells. *Immunity* (1997) **7**:493–504. doi:10.1016/S1074-7613(00)80371-4

11. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci U S A* (2010) **107**:10961–6. doi:10.1073/pnas.1005641107

12. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, Bucks C, Kane CM, Fallon PG, Pannell R, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* (2010) **464**:1367–1370. doi:10.1038/nature08900

13. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie ANJ, Mebius RE, et al. Innate lymphoid cells-a proposal for uniform nomenclature. *Nat Rev Immunol* (2013) **13**:145–149. doi:10.1038/nri3365

14. Juelke K, Romagnani C. Differentiation of human innate lymphoid cells (ILCs). *Curr Opin Immunol* (2016) **38**:75–85. doi:10.1016/J.COI.2015.11.005

15. Vonarbourg C, Diefenbach A. Multifaceted roles of interleukin-7 signaling for the development and function of innate lymphoid cells. *Semin Immunol* (2012) **24**:165–174. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22541512 [Accessed November 2, 2018]

16. Satoh-Takayama N, Lesjean-Pottier S, Vieira P, Sawa S, Eberl G, Vosshenrich CAJ, Di Santo JP. IL-7 and IL-15 independently program the differentiation of intestinal CD3 $^{-}$ NKp46 $^{+}$ cell subsets from Id2-dependent precursors. *J Exp Med* (2010) **207**:273–280. doi:10.1084/jem.20092029

17. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, Fabiunke C, Pfeifer D, Sexl V, Fonseca-Pereira D, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) **157**:340–356. doi:10.1016/j.cell.2014.03.030

18. Robinette ML, Bando JK, Song W, Ulland TK, Gilfillan S, Colonna M. IL-15 sustains IL-7R-independent ILC2 and ILC3 development. *Nat Commun* (2017) **8**:1–13. doi:10.1038/ncomms14601

19. Bouchery T, Kyle R, Camberis M, Shepherd A, Filbey K, Smith A, Harvie M, Painter G, Johnston K, Ferguson P, et al. ILC2s and T cells cooperate to ensure maintenance of M2 macrophages for lung immunity against hookworms. *Nat Commun* (2015) **6**:6970. doi:10.1038/ncomms7970

20. Rankin LC, Girard-Madoux MJH, Seillet C, Mielke LA, Kerdiles Y, Fenis A, Wieduwild E, Putoczki T, Mondot S, Lantz O, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat Immunol* (2016) **17**:179–186. doi:10.1038/ni.3332

21. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, Withers DR, Hugues S, Farrar MA, Reith W, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4^{+} T cells. *Science* (2015) **348**:1031–5. doi:10.1126/science.aaa4812

22. Kruglov AA, Grivennikov SI, Kuprash D V., Winsauer C, Prepens S, Seleznik GM, Eberl G, Littman DR, Heikenwalder M, Tumanov A V., et al. Nonredundant Function of Soluble LT 3 Produced by Innate Lymphoid Cells in Intestinal Homeostasis. *Science (80-)* (2013) **342**:1243–1246. doi:10.1126/science.1243364

23. Huang Q, Seillet C, Belz GT. Shaping innate lymphoid cell diversity. *Front Immunol* (2017) **8**:1–9. doi:10.3389/fimmu.2017.01569

24. Vivier E, van de Pavert SA, Cooper MD, Belz GT. The evolution of innate lymphoid cells. *Nat Immunol* (2016) **17**:790–4. doi:10.1038/ni.3459

25. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, Mullen AC, Gasink CR, Kaech SM, Miller JD, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* (2005) **6**:1236–1244. doi:10.1038/ni1268

26. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, Lindsten T, Reiner SL. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity* (2012) **36**:55–67. doi:10.1016/j.immuni.2011.11.016

27. Pikovskaya O, Chaix J, Rothman NJ, Collins A, Chen Y-H, Scipioni AM, Vivier E, Reiner SL. Cutting Edge: Eomesodermin Is Sufficient To Direct Type 1 Innate Lymphocyte Development into the Conventional NK Lineage. *J Immunol* (2016) **196**:1449–54. doi:10.4049/jimmunol.1502396

28. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* (1998) **9**:669–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9846488 [Accessed October 30, 2018]

29. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* (2000) **191**:771–80. doi:10.1084/JEM.191.5.771

30. Marcais A, Viel S, Grau M, Henry T, Marvel J, Walzer T. Regulation of mouse NK cell development

and function by cytokines. *Front Immunol* (2013) **4**:1–14. doi:10.3389/fimmu.2013.00450

31. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. (2008) doi:10.1038/ni1582

32. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* (2006) **6**:520–531. Available at: http://dx.doi.org/10.1038/nri1863

33. Walzer T, Bléry M, Chaix J, Fuseri N, Chasson L, Robbins SH, Jaeger S, André P, Gauthier L, Daniel L, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A* (2007) **104**:3384–9. doi:10.1073/pnas.0609692104

34. Werfel T, Uciechowski P, Tetteroo PA, Kurrle R, Deicher H, Schimdt R, Schmidt RE. Activation of cloned human natural killer cells via Fc gamma RIII. *J Immunol* (1989) **142**:1102–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2464637 [Accessed November 14, 2018]

35. Lord SJ, Rajotte R V, Korbutt GS, Bleackley RC. Granzyme B: a natural born killer. *Immunol Rev* (2003) **193**:31–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12752668

36. Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okamura H, Nakanishi K, Okumura K, Yagita H. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol* (1999) **163**:1906–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10438925 [Accessed November 2, 2018]

37. Wu H-J, Sawaya H, Binstadt B, Brickelmaier M, Blasius A, Gorelik L, Mahmood U, Weissleder R, Carulli J, Benoist C, et al. Inflammatory arthritis can be reined in by CpG-induced DC–NK cell cross talk. *J Exp Med* (2007) **204**:1911–1922. doi:10.1084/jem.20070285

38. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F. Induced recruitment of NK cells to lymph nodes provides IFN-[gamma] for TH1 priming. *Nat Immunol* (2004) **5**:1260–1265. Available at: http://dx.doi.org/10.1038/ni1138

39. Morandi B, Bougras G, Muller WA, Ferlazzo G, Münz C. NK cells of human secondary lymphoid tissues enhance T cell polarizationvia IFN-γ secretion. *Eur J Immunol* (2006) **36**:2394–2400. doi:10.1002/eji.200636290

40. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature* (2009) **457**:557–561. doi:10.1038/nature07665

41. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, Houchins JP, Miller S, Kang S-M, Norris PJ, et al. Expansion of a unique CD57+NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci* (2011) **108**:14725–14732. doi:10.1073/pnas.1110900108

42. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, Anasetti C, Weisdorf D, Miller JS. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol* (2012) **189**:5082–8. doi:10.4049/jimmunol.1201964

43. Cooper M a., Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56bright subset. *Blood* (2001) **97**:3146–3151. doi:10.1182/blood.V97.10.3146

44. Chan A, Hong D, Atzberger A, Filer AD, Buckley CD, Mcmichael A, Enver T, Bowness P, Kollnberger S. CD56bright human NK cells differentiate into CD56dim cells. *J Immunol* (2007) **179**:89–94.

45. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, Ratto G, Forte G, Carrega P, Lui G, et al. CD56brightCD16− Killer Ig-Like Receptor− NK Cells Display Longer Telomeres and

Acquire Features of CD56dim NK Cells upon Activation. *J Immunol* (2007) **178**:4947–4955. Available at: http://www.jimmunol.org/content/178/8/4947.abstract

46. Zhang J, Marotel M, Fauteux S, Mathieu A-L, Viel S, Marçais A, Walzer T. T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1. *Eur J Immunol* (2018)1–31. doi:10.1002/eji.201747299

47. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood* (2009) **113**:5488–5496. doi:10.1182/blood-2008-10-187179

48. Marquardt N, Wilk E, Pokoyski C, Schmidt RE, Jacobs R. Murine CXCR3+CD27bright NK cells resemble the human CD56bright NK-cell population. *Eur J Immunol* (2010) **40**:1428–1439. doi:10.1002/eji.200940056

49. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as Cell Surface Ligands for the Human DNAM-1 (CD226) Activating Molecule. *J Exp Med* (2003) **198**:557–567. doi:10.1084/jem.20030788

50. Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL, et al. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* (1996) **4**:573–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8673704 [Accessed November 1, 2018]

51. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, Yasui T, Kikutani H, Shibuya K, Shibuya A. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med* (2008) **205**:2959–64. doi:10.1084/jem.20081611

52. De Andrade LF, Smyth MJ, Martinet L. DNAM-1 control of natural killer cells functions through nectin and nectin-like proteins. *Immunol Cell Biol* (2014) **92**:237–244. doi:10.1038/icb.2013.95

53. Nabekura T, Kanaya M, Shibuya A, Fu G, Gascoigne NRJ, Lanier LL. Costimulatory Molecule DNAM-1 Is Essential for Optimal Differentiation of Memory Natural Killer Cells during Mouse Cytomegalovirus Infection. *Immunity* (2014) **40**:225–234. doi:10.1016/j.immuni.2013.12.011

54. Martinet L, Ferrari De Andrade L, Guillerey C, Lee JS, Liu J, Souza-Fonseca-Guimaraes F, Hutchinson DS, Kolesnik TB, Nicholson SE, Huntington ND, et al. DNAM-1 expression marks an alternative program of NK cell maturation. *Cell Rep* (2015) **11**:85–97. doi:10.1016/j.celrep.2015.03.006

55. Takeda K, Cretney E, Hayakawa Y, Ota T, Akiba H, Yagita H, Kinoshita K, Okumura K, Smyth MJ, Ogasawara K. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* (2005) **105**:2082–2089. doi:10.1182/blood-2004-08-3262

56. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, Bienvenu J, Henry T, Debien E, Hasan UA, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J Exp Med* (2014) **211**:563–77. doi:10.1084/jem.20131560

57. Spits H, Bernink JH, Lanier L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* (2016) **17**:758–764. doi:10.1038/ni.3482

58. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, Okumura K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* (2001) **7**:94–100. doi:10.1038/83416

59. Sonnenberg GF, Mjösberg J, Spits H, Artis D. SnapShot: Innate Lymphoid Cells. *Immunity* (2013)

39:622–622.e1. doi:10.1016/j.immuni.2013.08.021

60. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, Cella M, Colonna M. Intraepithelial Type 1 Innate Lymphoid Cells Are a Unique Subset of IL-12- and IL-15-Responsive IFN-γ-Producing Cells. *Immunity* (2013) **38**:769–781. doi:10.1016/J.IMMUNI.2013.02.010

61. Tang L, Peng H, Zhou J, Chen Y, Wei H, Sun R, Yokoyama WM, Tian Z. Differential phenotypic and functional properties of liver-resident NK cells and mucosal ILC1s. *J Autoimmun* (2016) **67**:29–35. doi:10.1016/j.jaut.2015.09.004

62. Weizman O-E El, Adams NM, Schuster IS, Krishna C, Pritykin Y, Lau C, Degli-Esposti MA, Leslie CS, Sun JC, O'Sullivan TE, et al. ILC1 Confer Early Host Protection at Initial Sites of Viral Infection. *Cell* (2017) **171**:795–808.e12. doi:10.1016/j.cell.2017.09.052

63. Mackay LK, Minnich M, Kragten NAM, Liao Y, Nota B, Seillet C, Zaid A, Man K, Preston S, Freestone D, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science (80-)* (2016) **352**:459–463. doi:10.1126/science.aad2035

64. Dunay IR, Diefenbach A. Group 1 innate lymphoid cells in *Toxoplasma gondii* infection. *Parasite Immunol* (2018) **40**:e12516. doi:10.1111/pim.12516

65. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, Hreggvidsdottir HS, Heinsbroek SE, Legrand N, Buskens CJ, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol* (2013) **14**:221–229. doi:10.1038/ni.2534

66. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Sušac B, Ling L, Leiner I, Pamer EG. Innate Immune Defenses Mediated by Two ILC Subsets Are Critical for Protection against Acute Clostridium difficile Infection. *Cell Host Microbe* (2015) **18**:27–37. doi:10.1016/J.CHOM.2015.06.011

67. O'Sullivan TE, Rapp M, Fan X, Weizman O-E, Bhardwaj P, Adams NM, Walzer T, Dannenberg AJ, Sun JC. Adipose-Resident Group 1 Innate Lymphoid Cells Promote Obesity-Associated Insulin Resistance. *Immunity* (2016) **45**:428–441. doi:10.1016/J.IMMUNI.2016.06.016

68. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngiow SF, Rautela J, Straube J, Waddell N, Blake SJ, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol* (2017) **18**:1004–1015. doi:10.1038/ni.3800

69. Dadi S, Chhangawala S, Whitlock BM, Franklin RA, Luo CT, Oh SA, Toure A, Pritykin Y, Huse M, Leslie CS, et al. Cancer Immunosurveillance by Tissue-Resident Innate Lymphoid Cells and Innate-like T Cells. *Cell* (2016) **164**:365–377. doi:10.1016/J.CELL.2016.01.002

70. Nixon BG, Li MO. Tissue-Resident Cytolytic Innate Lymphocytes in Cancer. *J Immunol* (2018) **200**:408–414. doi:10.4049/jimmunol.1701124

71. Mami-Chouaib F, Strutt TM, Seddon B, Li MO, Chou C. Tissue-Resident Lymphocytes Across Innate and Adaptive Lineages. *Front Immunol | www.frontiersin.org* (2018) **9**:2104. doi:10.3389/fimmu.2018.02104

72. Huang Y, Paul WE. Inflammatory group 2 innate lymphoid cells. *Int Immunol* (2016) **28**:23–8. doi:10.1093/intimm/dxv044

73. Artis D, Spits H. The biology of innate lymphoid cells. *Nature* (2015) **517**:293–301. doi:10.1038/nature14189

74. Monticelli LA, Osborne LC, Noti M, Tran S V, Zaiss DMW, Artis D. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions. *Proc Natl Acad Sci U S A* (2015) **112**:10762–7. doi:10.1073/pnas.1509070112

75. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, Angelosanto JM, Laidlaw BJ, Yang CY, Sathaliyawala T, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* (2011) **12**:1045–1054. doi:10.1038/ni.2131

76. Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, Thome JJ, Farber DL, Lutfy K, Seale P, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* (2015) **519**:242–246. doi:10.1038/nature14115

77. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, Mantovani A, Kopan R, Bradfield CA, Newberry RD, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol* (2011) **13**:144–51. doi:10.1038/ni.2187

78. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Bérard M, Kleinschek M, Cua D, Di Santo JP, Eberl G. RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol* (2011) **12**:320–326. doi:10.1038/ni.2002

79. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, Shibata N, Grunberg S, Sinha R, Zahm AM, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* (2012) **336**:1321–5. doi:10.1126/science.1222551

80. Satoh-Takayama N, Vosshenrich CAJ, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, Mention J-J, Thiam K, Cerf-Bensussan N, Mandelboim O, et al. Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46+ Cells that Provide Innate Mucosal Immune Defense. *Immunity* (2008) **29**:958–970. doi:10.1016/J.IMMUNI.2008.11.001

81. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol* (2011) **12**:383–390. doi:10.1038/ni.2025

82. Taube C, Tertilt C, Gyülveszi G, Dehzad N, Kreymborg K, Schneeweiss K, Michel E, Reuter S, Renauld J-C, Arnold-Schild D, et al. IL-22 Is Produced by Innate Lymphoid Cells and Limits Inflammation in Allergic Airway Disease. *PLoS One* (2011) **6**:e21799. doi:10.1371/journal.pone.0021799

83. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A. RORγt and commensal microflora are required for the differentiation of mucosal interleukin 22–producing NKp46+ cells. *Nat Immunol* (2009) **10**:83–91. doi:10.1038/ni.1684

84. Luci C, Reynders A, Ivanov II, Cognet C, Chiche L, Chasson L, Hardwigsen J, Anguiano E, Banchereau J, Chaussabel D, et al. Influence of the transcription factor RORγt on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* (2009) **10**:75–82. doi:10.1038/ni.1681

85. Eisenring M, vom Berg J, Kristiansen G, Saller E, Becher B. IL-12 initiates tumor rejection via lymphoid tissue–inducer cells bearing the natural cytotoxicity receptor NKp46. *Nat Immunol* (2010) **11**:1030–1038. doi:10.1038/ni.1947

86. Carrega P, Loiacono F, Di Carlo E, Scaramuccia A, Mora M, Conte R, Benelli R, Spaggiari GM, Cantoni C, Campana S, et al. NCR+ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. *Nat Commun* (2015) **6**:8280. doi:10.1038/ncomms9280

87. Nussbaum K, Burkhard SH, Ohs I, Mair F, Klose CSN, Arnold SJ, Diefenbach A, Tugues S, Becher B. Tissue microenvironment dictates the fate and tumor-suppressive function of type 3 ILCs. *J Exp Med* (2017) **214**:2331–2347. doi:10.1084/jem.20162031

88. Meier D, Bornmann C, Chappaz S, Schmutz S, Otten LA, Ceredig R, Acha-Orbea H, Finke D. Ectopic Lymphoid-Organ Development Occurs through Interleukin 7-Mediated Enhanced Survival of Lymphoid-Tissue-Inducer Cells. *Immunity* (2007) **26**:643–654. doi:10.1016/j.immuni.2007.04.009

89. Eberl G, Marmon S, Sunshine M-J, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORγt in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* (2004) **5**:64–73. doi:10.1038/ni1022

90. Yoshida H, Naito A, Inoue J-I, Satoh M, Santee-Cooper SM, Ware CF, Togawa A, Nishikawa S, Nishikawa S-I. Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. *Immunity* (2002) **17**:823–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12479827 [Accessed November 4, 2018]

91. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara-Astiaso D, Levy M, Salame TM, Weiner A, David E, et al. The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell* (2016) **166**:1231–1246.e13. doi:10.1016/j.cell.2016.07.043

92. Cortez VS, Cervantes-Barragan L, Robinette ML, Bando JK, Wang Y, Geiger TL, Gilfillan S, Fuchs A, Vivier E, Sun JC, et al. Transforming Growth Factor-β Signaling Guides the Differentiation of Innate Lymphoid Cells in Salivary Glands. *Immunity* (2016) **44**:1127–1139. doi:10.1016/j.immuni.2016.03.007

93. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, Flach M, Bengsch B, Thimme R, Hölscher C, et al. Regulated Expression of Nuclear Receptor RORγt Confers Distinct Functional Fates to NK Cell Receptor-Expressing RORγt+ Innate Lymphocytes. *Immunity* (2010) **33**:736–751. doi:10.1016/j.immuni.2010.10.017

94. Klose CSN, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, Göppert N, Croxford AL, Waisman A, Tanriver Y, et al. A T-bet gradient controls the fate and function of CCR6−RORγt+ innate lymphoid cells. *Nature* (2013) **494**:261–265. doi:10.1038/nature11813

95. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, Munneke JM, Hazenberg MD, Villaudy J, Buskens CJ, et al. Interleukin-12 and -23 Control Plasticity of CD127+ Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. *Immunity* (2015) **43**:146–160. doi:10.1016/J.IMMUNI.2015.06.019

96. Sojka DK, Plougastel-Douglas B, Yang L, Pak-Wittel MA, Artyomov MN, Ivanova Y, Zhong C, Chase JM, Rothman PB, Yu J, et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *Elife* (2014) **2014**:1–21. doi:10.7554/eLife.01659

97. Vosshenrich CAJ, García-Ojeda ME, Samson-Villéger SI, Pasqualetto V, Enault L, Goff OR Le, Corcuff E, Guy-Grand D, Rocha B, Cumano A, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* (2006) **7**:1217–1224. doi:10.1038/ni1395

98. Cortez VS, Fuchs A, Cella M, Gilfillan S, Colonna M. Cutting edge: Salivary gland NK cells develop independently of Nfil3 in steady-state. *J Immunol* (2014) **192**:4487–91. doi:10.4049/jimmunol.1303469

99. Montaldo E, Vacca P, Chiossone L, Croxatto D, Loiacono F, Martini S, Ferrero S, Walzer T, Moretta L, Mingari MC. Unique Eomes+NK cell subsets are present in uterus and decidua during early pregnancy. *Front Immunol* (2016) **6**:1–11. doi:10.3389/fimmu.2015.00646

100. Peng H, Tian Z. Re-examining the origin and function of liver-resident NK cells. *Trends Immunol*

(2015) **36**:293–299. doi:10.1016/j.it.2015.03.006

101. Filipovic I, Chiossone L, Vacca P, Hamilton RS, Ingegnere T, Doisne J-M, Hawkes DA, Mingari MC, Sharkey AM, Moretta L, et al. Molecular definition of group 1 innate lymphoid cells in the mouse uterus. *Nat Commun* (2018) **9**:4492. doi:10.1038/s41467-018-06918-3

102. Crotta S, Gkioka A, Male V, Duarte JH, Davidson S, Nisoli I, Brady HJM, Wack A. The transcription factor E4BP4 is not required for extramedullary pathways of NK cell development. *J Immunol* (2014) **192**:2677–88. doi:10.4049/jimmunol.1302765

103. Seillet C, Huntington ND, Gangatirkar P, Axelsson E, Minnich M, Brady HJM, Busslinger M, Smyth MJ, Belz GT, Carotta S. Differential requirement for Nfil3 during NK cell development. *J Immunol* (2014) **192**:2667–76. doi:10.4049/jimmunol.1302605

104. Diefenbach A, Colonna M, Koyasu S. Development, Differentiation, and Diversity of Innate Lymphoid Cells. *Immunity* (2014) **41**:354–365. doi:10.1016/J.IMMUNI.2014.09.005

105. Gasteiger G, Fan X, Dikiy S, Lee SY, Alexander Y, Program I, Sloan- M. Tissue residency of innate lymphoid cells in lymphoid and non- lymphoid organs. *Science (80-)* (2015) **350**:981–985. doi:10.1126/science.aac9593.Tissue

106. Huang Y, Mao K, Chen X, Sun M-A, Kawabe T, Li W, Usher N, Zhu J, Urban JF, Paul WE, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* (2018) **359**:114–119. doi:10.1126/science.aam5809

107. Proudfoot AEI. Chemokine receptors: multifaceted therapeutic targets. *Nat Rev Immunol* (2002) **2**:106–115. doi:10.1038/nri722

108. Metzemaekers M, Vanheule V, Janssens R, Struyf S, Proost P. Overview of the mechanisms that may contribute to the non-redundant activities of interferon-inducible CXC chemokine receptor 3 ligands. *Front Immunol* (2018) **8**: doi:10.3389/fimmu.2017.01970

109. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Molecular biology of the cell*. Garland Science (2002).

110. Tawil NJ, Houde M, Blacher R, Esch F, Reichardt LF, Turner DC, Carbonetto S. Alpha 1 beta 1 integrin heterodimer functions as a dual laminin/collagen receptor in neural cells. *Biochemistry* (1990) **29**:6540–4. doi:10.1021/BI00479A028

111. Hadley GA, Bartlett ST, Via CS, Rostapshova EA, Moainie S. The epithelial cell-specific integrin, CD103 (alpha E integrin), defines a novel subset of alloreactive CD8+ CTL. *J Immunol* (1997) **159**:3748–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9378961 [Accessed November 6, 2018]

112. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, Lipp M. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* (1999) **99**:23–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10520991 [Accessed November 6, 2018]

113. Okada T, Cyster JG. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. *J Immunol* (2007) **178**:2973–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17312142 [Accessed November 7, 2018]

114. Hamann I, Unterwalder N, Cardona AE, Meisel C, Zipp F, Ransohoff RM, Infante-Duarte C. Analyses of phenotypic and functional characteristics of CX3CR1-expressing natural killer cells. *Immunology* (2011) **133**:62–73. doi:10.1111/j.1365-2567.2011.03409.x

115. Hertwig L, Hamann I, Romero-Suarez S, Millward JM, Pietrek R, Chanvillard C, Stuis H, Pollok K, Ransohoff RM, Cardona AE, et al. CX3CR1-dependent recruitment of mature NK cells into the

central nervous system contributes to control autoimmune neuroinflammation. *Eur J Immunol* (2016) **46**:1984–1996. doi:10.1002/eji.201546194

116. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol* (2000) **1**:298–304. doi:10.1038/79738

117. Paust S, Gill HS, Wang B-Z, Flynn MP, Moseman EA, Senman B, Szczepanik M, Telenti A, Askenase PW, Compans RW, et al. Critical role for the chemokine receptor CXCR6 in NK cell–mediated antigen-specific memory of haptens and viruses. *Nat Immunol* (2010) **11**:1127–1135. doi:10.1038/ni.1953

118. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, et al. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* (1998) **187**:2009–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9625760 [Accessed November 6, 2018]

119. Farber JM. A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. *Proc Natl Acad Sci U S A* (1990) **87**:5238–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2115167 [Accessed November 6, 2018]

120. Luster AD. Chemokines — Chemotactic Cytokines That Mediate Inflammation. *N Engl J Med* (1998) **338**:436–445. doi:10.1056/NEJM199802123380706

121. Nakajima C, Mukai T, Yamaguchi N, Morimoto Y, Park W-R, Iwasaki M, Gao P, Ono S, Fujiwara H, Hamaoka T. Induction of the chemokine receptor CXCR3 on TCR-stimulated T cells: dependence on the release from persistent TCR-triggering and requirement for IFN-γ stimulation. *Eur J Immunol* (2002) **32**:1792. doi:10.1002/1521-4141(200206)32:6<1792::AID-IMMU1792>3.0.CO;2-0

122. Karin N, Razon H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. (2018) doi:10.1016/j.cyto.2018.02.012

123. Shiow LR, Rosen DB, Brdičková N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* (2006) **440**:540–544. doi:10.1038/nature04606

124. Cyster JG, Schwab SR. Sphingosine-1-Phosphate and Lymphocyte Egress from Lymphoid Organs. *Annu Rev Immunol* (2012) **30**:69–94. doi:10.1146/annurev-immunol-020711-075011

125. Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol* (2017) **47**:946–953. doi:10.1002/eji.201646837

126. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* (1948) **29**:58–69. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18865105 [Accessed November 6, 2018]

127. Greene HN. The Transplantation of Tumors to the Brains of Heterologous Species*. (1951). Available at: http://cancerres.aacrjournals.org/content/canres/11/7/529.full.pdf [Accessed November 6, 2018]

128. Takeshita Y, Ransohoff RM. Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models. *Immunol Rev* (2012) **248**:228–239. doi:10.1111/j.1600-065X.2012.01127.x

129. Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. *Nat Immunol* (2017) **18**:123–131. doi:10.1038/ni.3666

130. Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. *J Clin Invest* (2010) **120**:1368–79. doi:10.1172/JCI41911

131. Schwartz M, Kipnis J, Rivest S, Prat A. How do immune cells support and shape the brain in health, disease, and aging? *J Neurosci* (2013) **33**:17587–96. doi:10.1523/JNEUROSCI.3241-13.2013

132. Schwartz M, Raposo C. Protective Autoimmunity: A Unifying Model for the Immune Network Involved in CNS Repair. *Neurosci* (2014) **20**:343–358. doi:10.1177/1073858413516799

133. Kida S, Pantazis A, Weller RO. CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. *Neuropathol Appl Neurobiol* (1993) **19**:480–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7510047 [Accessed November 7, 2018]

134. Johnston M, Zakharov A, Papaiconomou C, Salmasi G, Armstrong D. Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. *Cerebrospinal Fluid Res* (2004) **1**:2. doi:10.1186/1743-8454-1-2

135. Martini F, Timmons M, Tallitsch R. *Human Anatomy*. Eight edit. Pearson (2015).

136. Engelhardt B, Sorokin L. The blood–brain and the blood–cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol* (2009) **31**:497–511. doi:10.1007/s00281-009-0177-0

137. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood–brain barriers. *Trends Immunol* (2012) **33**:579–589. doi:10.1016/J.IT.2012.07.004

138. McCandless EE, Wang Q, Woerner BM, Harper JM, Klein RS. CXCL12 Limits Inflammation by Localizing Mononuclear Infiltrates to the Perivascular Space during Experimental Autoimmune Encephalomyelitis. *J Immunol* (2006) **177**:8053–8064. doi:10.4049/jimmunol.177.11.8053

139. Müller M, Carter SL, Hofer MJ, Manders P, Getts DR, Getts MT, Dreykluft A, Lu B, Gerard C, King NJC, et al. CXCR3 Signaling Reduces the Severity of Experimental Autoimmune Encephalomyelitis by Controlling the Parenchymal Distribution of Effector and Regulatory T Cells in the Central Nervous System. *J Immunol* (2007) **179**:2774–2786. doi:10.4049/jimmunol.179.5.2774

140. Greter M, Heppner FL, Lemos MP, Odermatt BM, Goebels N, Laufer T, Noelle RJ, Becher B. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat Med* (2005) **11**:328–334. doi:10.1038/nm1197

141. Miller SD, McMahon EJ, Schreiner B, Bailey SL. Antigen Presentation in the CNS by Myeloid Dendritic Cells Drives Progression of Relapsing Experimental Autoimmune Encephalomyelitis. *Ann N Y Acad Sci* (2007) **1103**:179–191. doi:10.1196/annals.1394.023

142. Bartholomäus I, Kawakami N, Odoardi F, Schläger C, Miljkovic D, Ellwart JW, Klinkert WEF, Flügel-Koch C, Issekutz TB, Wekerle H, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* (2009) **462**:94–98. doi:10.1038/nature08478

143. Baruch K, Ron-Harel N, Gal H, Deczkowska A, Shifrut E, Ndifon W, Mirlas-Neisberg N, Cardon M, Vaknin I, Cahalon L, et al. CNS-specific immunity at the choroid plexus shifts toward destructive Th2 inflammation in brain aging. *PNAS* (2013) **110**:2264–2269. doi:10.1073/pnas.1211270110

144. Kunis G, Baruch K, Rosenzweig N, Kertser A, Miller O, Berkutzki T, Schwartz M. IFN-γ-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair. *Brain* (2013) **136**:3427–3440. doi:10.1093/brain/awt259

145. Kivisäkk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H,

Staugaitis SM, et al. Human cerebrospinal fluid central memory CD4 + T cells: Evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc Natl Acad Sci* (2003) **100**:8389–8394. doi:10.1073/pnas.1433000100

146. Hamann I, Dörr J, Glumm R, Chanvillard C, Janssen A, Millward JM, Paul F, Ransohoff RM, Infante-Duarte C. Characterization of natural killer cells in paired CSF and blood samples during neuroinflammation. *J Neuroimmunol* (2013) **254**:165–169. doi:http://dx.doi.org/10.1016/j.jneuroim.2012.08.009

147. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, et al. Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science (80-)* (2010) **330**:841–845. doi:10.1126/science.1194637

148. Nayak D, Roth TL, McGavern DB. Microglia Development and Function. *Annu Rev Immunol* (2014) **32**:367–402. doi:10.1146/annurev-immunol-032713-120240

149. Greter M, Lelios I, Croxford AL. Microglia versus myeloid cell nomenclature during brain inflammation. *Front Immunol* (2015) **6**:1–7. doi:10.3389/fimmu.2015.00249

150. Poli A, Kmiecik J, Domingues O, Hentges F, Blery M, Chekenya M, Boucraut J, Zimmer J. NK Cells in Central Nervous System Disorders. *J Immunol* (2013) **190**:5355–5362. doi:10.4049/jimmunol.1203401

151. Korin B, Ben-Shaanan TL, Schiller M, Dubovik T, Azulay-Debby H, Boshnak NT, Koren T, Rolls A. High-dimensional, single-cell characterization of the brain's immune compartment. *Nat Neurosci* (2017) **20**:1300–1309. doi:10.1038/nn.4610

152. Raffel J, Wakerley B, Nicholas R. Multiple sclerosis. *Medicine (Baltimore)* (2016) **44**:537–541. doi:10.1016/J.MPMED.2016.06.005

153. Petersen G, Wittmann R, Arndt V, Göpffarth D. Epidemiologie der Multiplen Sklerose in Deutschland. *Nervenarzt* (2014) **85**:990–998. doi:10.1007/s00115-014-4097-4

154. Compston A, Coles A. Multiple Sclerosis. *Lancet* (2008) **372**:1502–17. doi:10.1016/S0140-6736(08)61620-7

155. Sawcer S, Ban M, Maranian M, Yeo TW, Compston A, Kirby A, Daly MJ, De Jager PL, Walsh E, Lander ES, et al. A High-Density Screen for Linkage in Multiple Sclerosis. *Am J Hum Genet* (2005) **77**:454–467. doi:10.1086/444547

156. International Multiple Sclerosis Genetics Consortium, Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, de Bakker PIW, Gabriel SB, Mirel DB, et al. Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study. *N Engl J Med* (2007) **357**:851–862. doi:10.1056/NEJMoa073493

157. International Multiple Sclerosis Genetics Consortium S, Wellcome Trust Case Control Consortium 2 G, Sawcer S, Hellenthal G, Pirinen M, Spencer CCA, Patsopoulos NA, Moutsianas L, Dilthey A, Su Z, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* (2011) **476**:214–9. doi:10.1038/nature10251

158. Mumford CJ, Wood NW, Kellar-Wood H, Thorpe JW, Miller DH, Compston DA. The British Isles survey of multiple sclerosis in twins. *Neurology* (1994) **44**:11–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8290043 [Accessed November 8, 2018]

159. Willer CJ, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC, Group TCCS. Twin concordance and sibling recurrence rates in multiple sclerosis. *Proc Natl Acad Sci* (2003) **100**:12877–12882. doi:10.1073/pnas.1932604100

160. Ristori G, Cannoni S, Stazi MA, Vanacore N, Cotichini R, Alfò M, Pugliatti M, Sotgiu S, Solaro C,

Bomprezzi R, et al. Multiple sclerosis in twins from continental Italy and Sardinia: A nationwide study. *Ann Neurol* (2006) **59**:27–34. doi:10.1002/ana.20683

161. Koduah P, Paul F, Dörr J-M. Vitamin D in the prevention, prediction and treatment of neurodegenerative and neuroinflammatory diseases. *EPMA J* (2017) **8**:313–325. doi:10.1007/s13167-017-0120-8

162. Fernández-Menéndez S, Fernández-Morán M, Fernández-Vega I, Pérez-Álvarez A, Villafani-Echazú J. Epstein-Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *J Neurol Sci* (2016) **361**:213–9. doi:10.1016/j.jns.2016.01.013

163. Pender MP. The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *Neuroscientist* (2011) **17**:351–67. doi:10.1177/1073858410381531

164. Laurence M, Benito-León J. Epstein–Barr virus and multiple sclerosis: Updating Pender's hypothesis. *Mult Scler Relat Disord* (2017) **16**:8–14. doi:10.1016/J.MSARD.2017.05.009

165. Buljevac D, Flach HZ, Hop WCJ, Hijdra D, Laman JD, Savelkoul HFJ, van der Meché FGA, van Doorn PA, Hintzen RQ. Prospective study on the relationship between infections and multiple sclerosis exacerbations. *Brain* (2002) **125**:952–960. doi:10.1093/brain/awf098

166. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* (2002) **2**:85–95. doi:10.1038/nri724

167. Shahi SK, Freedman SN, Mangalam AK, Shahi K. Gut Microbes Gut microbiome in multiple sclerosis: The players involved and the roles they play Gut microbiome in multiple sclerosis: The players involved and the roles they play. (2017) doi:10.1080/19490976.2017.1349041

168. Yadav SK, Mindur JE, Ito K, Dhib-Jalbut S. Advances in the immunopathogenesis of multiple sclerosis. *Curr Opin Neurol* (2015) **28**:206–219. doi:10.1097/WCO.0000000000000205

169. Wekerle H. B cells in multiple sclerosis. *Autoimmunity* (2017) **50**:57–60. doi:10.1080/08916934.2017.1281914

170. Funaro M, Messina M, Shabbir M, Wright P, Najjar S, Tabansky I, Stern JNH. The role of B cells in multiple sclerosis: more than antibodies. *Discov Med* (2016) **22**:251–255. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28009967 [Accessed November 10, 2018]

171. McDonald WI, Compston a, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol* (2001) **50**:121–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11456302

172. Fisniku LK, Brex PA, Altmann DR, Miszkiel KA, Benton CE, Lanyon R, Thompson AJ, Miller DH. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain* (2008) **131**:808–817. doi:10.1093/brain/awm329

173. Kurtzke J. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* (1983) **33**:1444–52.

174. Stuart G, Krikorian KS. The Neuro-Paralytic Accidents of Anti-Rabies Treatment. *Ann Trop Med Parasitol* (1928) **22**:327–377. doi:10.1080/00034983.1928.11684582

175. Lassmann H, Bradl M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol* (2017) **133**:223–244. doi:10.1007/s00401-016-1631-4

176. Rivers TM, Sprunt DH, Berry GP. Obaservations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J Exp Med* (1933) **58**:39–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19870180 [Accessed November 11, 2018]

177. Simmons SB, Pierson ER, Lee SY, Goverman JM. Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol* (2013) **34**:410–422. doi:10.1016/J.IT.2013.04.006

178. Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. Animal models of Multiple Sclerosis. *Eur J Pharmacol* (2015) **759**:182–191. doi:10.1016/J.EJPHAR.2015.03.042

179. Pierson E, Simmons SB, Castelli L, Goverman JM. Mechanisms regulating regional localization of inflammation during CNS autoimmunity. *Immunol Rev* (2012) **248**:205–215. doi:10.1111/j.1600-065X.2012.01126.x

180. Ransohoff RM. Animal models of multiple sclerosis: the good, the bad and the bottom line. (2012) doi:10.1038/nn.3168

181. Praet J, Guglielmetti C, Berneman Z, Van der Linden A, Ponsaerts P. Cellular and molecular neuropathology of the cuprizone mouse model: Clinical relevance for multiple sclerosis. *Neurosci Biobehav Rev* (2014) **47**:485–505. doi:10.1016/j.neubiorev.2014.10.004

182. Benczur M, Petrányl GG, Pálffy G, Varga M, Tálas M, Kotsy B, Földes I, Hollán SR. Dysfunction of natural killer cells in multiple sclerosis: a possible pathogenetic factor. *Clin Exp Immunol* (1980) **39**:657–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6155232 [Accessed November 13, 2018]

183. Merrill J, Jondal M, Seeley J, Ullberg M, Sidén a. Decreased NK killing in patients with multiple sclerosis: an analysis on the level of the single effector cell in peripheral blood and cerebrospinal fluid in relation to the activity in the disease. *Clin Exp Immunol* (1982) **47**:419–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6176377 [Accessed November 13, 2018]

184. Neighbour PA, Grayzel AI, Miller AE. Endogenous and interferon-augmented natural killer cell activity of human peripheral blood mononuclear cells in vitro. Studies of patients with multiple sclerosis, systemic lupus erythematosus or rheumatoid arthritis. *Clin Exp Immunol* (1982) **49**:11–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6181920 [Accessed November 13, 2018]

185. Oger J, Kastrukoff LF, Li DK, Paty DW. Multiple sclerosis: in relapsing patients, immune functions vary with disease activity as assessed by MRI. *Neurology* (1988) **38**:1739–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3185908 [Accessed November 13, 2018]

186. Kastrukoff LF, Morgan NG, Zecchini D, White R, Petkau AJ, Satoh J, Paty DW. A role for natural killer cells in the immunopathogenesis of multiple sclerosis. *J Neuroimmunol* (1998) **86**:123–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9663557 [Accessed November 13, 2018]

187. Kastrukoff LF, Lau A, Wee R, Zecchini D, White R, Paty DW. Clinical relapses of multiple sclerosis are associated with 'novel' valleys in natural killer cell functional activity. *J Neuroimmunol* (2003) **145**:103–114. doi:http://dx.doi.org/10.1016/j.jneuroim.2003.10.001

188. Lünemann A, Tackenberg B, DeAngelis T, da Silva RB, Messmer B, Vanoaica LD, Miller A, Apatoff B, Lublin FD, Lünemann JD, et al. Impaired IFN-γ production and proliferation of NK cells in multiple sclerosis. *Int Immunol* (2011) **23**:139–148. doi:10.1093/intimm/dxq463

189. Infante-duarte C, Weber A, Krätzschmar J, Prozorovski T, Pikol S, Hamann I, Bellmann-strobl J, Aktas O, Dörr J, Wuerfel J, et al. Frequency of blood CX3CR1.positive natural killer cells correlates with disease activity in multiple sclerosis patients. *FASEB J* (2005) **19**:

190. Airas L, Saraste M, Rinta S, Elovaara I, Huang Y-H, Wiendl H, Finnish Multiple Sclerosis and Pregnancy Study Group. Immunoregulatory factors in multiple sclerosis patients during and after pregnancy: relevance of natural killer cells. *Clin Exp Immunol* (2007) **151**:235–243. doi:10.1111/j.1365-2249.2007.03555.x

191. Saraste M, Irjala H, Airas L. Expansion of CD56Bright natural killer cells in the peripheral blood of multiple sclerosis patients treated with interferon-beta. *Neurol Sci* (2007) **28**:121–6. doi:10.1007/s10072-007-0803-3

192. Bielekova B, Catalfamo M, Reichert-scrivner S, Packer A, Cerna M, Waldmann TA, Mcfarland H, Henkart PA, Martin R. Regulatory CD56 bright natural killer cells mediate immunomodulatory effects of IL-2Ra-targeted therapy (daclizumab) in multiple sclerosis. (2006) **103**:5941–5946.

193. Gross CC, Schulte-Mecklenbeck A, Rünzi A, Kuhlmann T, Posevitz-Fejfár A, Schwab N, Schneider-Hohendorf T, Herich S, Held K, Konjević M, et al. Impaired NK-mediated regulation of T-cell activity in multiple sclerosis is reconstituted by IL-2 receptor modulation. *Proc Natl Acad Sci* (2016)201524924. doi:10.1073/pnas.1524924113

194. Laroni A, Armentani E, Kerlero de Rosbo N, Ivaldi F, Marcenaro E, Sivori S, Gandhi R, Weiner HL, Moretta A, Mancardi GL, et al. Dysregulation of regulatory CD56 bright NK cells/T cells interactions in multiple sclerosis. *J Autoimmun* (2016) **72**:8–18. doi:10.1016/j.jaut.2016.04.003

195. Anolik JH, Campbell D, Felgar RE, Young F, Sanz I, Rosenblatt J, Looney RJ. The relationship of Fc?RIIIa genotype to degree of B cell depletion by rituximab in the treatment of systemic lupus erythematosus. *Arthritis Rheum* (2003) **48**:455–459. doi:10.1002/art.10764

196. Hu Y, Turner MJ, Shields J, Gale MS, Hutto E, Roberts BL, Siders WM, Kaplan JM. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology* (2009) **128**:260–270. doi:10.1111/j.1365-2567.2009.03115.x

197. Shi F-D, Takeda K, Akira S, Sarvetnick N, Ljunggren H-G. IL-18 Directs Autoreactive T Cells and Promotes Autodestruction in the Central Nervous System Via Induction of IFN-γ by NK Cells. *J Immunol* (2000) **165**:3099 LP-3104. Available at: http://www.jimmunol.org/content/165/6/3099.abstract

198. Winkler-Pickett R, Young HA, Cherry JM, Diehl J, Wine J, Back T, Bere WE, Mason AT, Ortaldo JR. In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses. *J Immunol* (2008) **180**:4495–506. doi:10.4049/JIMMUNOL.180.7.4495

199. Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* (1997) **186**:1677–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9362528 [Accessed November 14, 2018]

200. Xu W, Fazekas G, Hara H, Tabira T. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J Neuroimmunol* (2005) **163**:24–30. doi:http://dx.doi.org/10.1016/j.jneuroim.2005.02.011

201. Hao J, Campagnolo D, Liu R, Piao W, Shi S, Hu B, Xiang R, Zhou Q, Vollmer T, Van Kaer L, et al. Interleukin-2/interleukin-2 antibody therapy induces target organ natural killer cells that inhibit central nervous system inflammation. *Ann Neurol* (2011) **69**:721–34. doi:10.1002/ana.22339

202. Lu L, Ikizawa K, Hu D, Werneck MBF, Wucherpfennig KW, Cantor H. Regulation of Activated CD4+ T Cells by NK Cells via the Qa-1–NKG2A Inhibitory Pathway. *Immunity* (2007) **26**:593–604. doi:10.1016/J.IMMUNI.2007.03.017

203. Leavenworth JW, Schellack C, Kim H-J, Lu L, Spee P, Cantor H. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')2. *Proc Natl Acad Sci U S A* (2010) **107**:2562–7. doi:10.1073/pnas.0914732107

204. Huang D, Shi F-D, Jung S, Pien GC, Wang J, Salazar-Mather TP, He TT, Weaver JT, Ljunggren H-G, Biron CA, et al. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. (2006)

20:896–905. doi:10.1096/fj.05-5465com

205. Hao J, Liu R, Piao W, Zhou Q, Vollmer TL, Campagnolo DI, Xiang R, La Cava A, Van Kaer L, Shi F-D. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *J Exp Med* (2010) **207**:1907–1921. doi:10.1084/jem.20092749

206. Liu Q, Sanai N, Jin W-N, La Cava A, Van Kaer L, Shi F-D. Neural stem cells sustain natural killer cells that dictate recovery from brain inflammation. *Nat Neurosci* (2016)1–12. doi:10.1038/nn.4211

207. Kwong B, Rua R, Gao Y, Flickinger J, Wang Y, Kruhlak MJ, Zhu J, Vivier E, McGavern DB, Lazarevic V. T-bet-dependent NKp46 + innate lymphoid cells regulate the onset of T H 17-induced neuroinflammation. *Nat Immunol* (2017) **18**:1117–1127. doi:10.1038/ni.3816

208. Ormerod MG. *Flow cytometry- A Basic Introduction*.

209. Bernardini G, Antonangeli F, Bonanni V, Santoni A. Dysregulation of chemokine/chemokine receptor axes and NK cell tissue localization during diseases. *Front Immunol* (2016) **7**:1–9. doi:10.3389/fimmu.2016.00402

210. Pesce S, Moretta L, Moretta A, Marcenaro E. Human NK cell subsets redistribution in pathological conditions: A role for CCR7 receptor. *Front Immunol* (2016) **7**: doi:10.3389/fimmu.2016.00414

211. Peng H, Tian Z. NK cell trafficking in health and autoimmunity:a comprehensive review. *Clin Rev Allergy Immunol* (2014) **47**:119–127. doi:10.1007/s12016-013-8400-0

212. Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Rottman J, Sellebjerg F, Strieter RM, et al. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* (1999) **103**:807–15. doi:10.1172/JCI5150

213. Müller M, Carter S, Hofer MJ, Campbell IL. Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity - A tale of conflict and conundrum. *Neuropathol Appl Neurobiol* (2010) **36**:368–387. doi:10.1111/j.1365-2990.2010.01089.x

214. Colvin RA, Campanella GS V, Sun J, Luster AD. Intracellular domains of CXCR3 that mediate CXCL9, CXCL10, and CXCL11 function. *J Biol Chem* (2004) **279**: doi:10.1074/jbc.M403595200

215. Colvin RA, Campanella GS V, Manice LA, Luster AD. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Mol Cell Biol* (2006) **26**:5838–49. doi:10.1128/MCB.00556-06

216. Karin N, Wildbaum G, Thelen M. Biased signaling pathways via CXCR3 control the development and function of CD4+ T cell subsets. *J Leukoc Biol* (2016) **99**:857–862. doi:10.1189/jlb.2MR0915-441R

217. Zohar Y, Wildbaum G, Novak R, Salzman AL, Thelen M, Alon R, Barsheshet Y, Karp CL, Karin N. CXCL11-dependent induction of FOXP3- negative regulatory T cells suppresses autoimmune encephalomyelitis. *J Clin Investig* (2014) **124**: doi:10.1172/JCI71951.and

218. Shechter R, London A, Schwartz M. Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. *Nat Rev Immunol* (2013) **13**:206–218. doi:10.1038/nri3391

219. Alt C, Laschinger M, Engelhardt B. Functional expression of the lymphoid chemokines CCL19 (ELC) and CCL 21 (SLC) at the blood-brain barrier suggests their involvement in G-protein-dependent lymphocyte recruitment into the central nervous system during experimental autoimmune encephalomyelitis. *Eur J Immunol* (2002) **32**:2133. doi:10.1002/1521-

4141(200208)32:8<2133::AID-IMMU2133>3.0.CO;2-W

220. Mayol K, Biajoux V, Marvel J, Balabanian K, Walzer T. Sequential desensitization of CXCR4 and S1P5 controls natural killer cell trafficking. *Blood* (2011) **118**:4863–71. doi:10.1182/blood-2011-06-362574

221. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM V. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci* (2011) **14**:1142–1149. doi:10.1038/nn.2887

222. Freedman NJ, Lefkowitz RJ. Desensitization of G protein-coupled receptors. *Recent Prog Horm Res* (1996) **51**:319-51; discussion 352–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8701085 [Accessed December 3, 2018]

223. Moro O, Lameh J, Sadbe W. Serine-and Threonine-rich Domain Regulates Internalization of Muscarinic Cholinergic Receptors*. (1993). Available at: http://www.jbc.org/content/268/10/6862.full.pdf [Accessed December 3, 2018]

224. Meiser A, Mueller A, Wise EL, McDonagh EM, Petit SJ, Saran N, Clark PC, Williams TJ, Pease JE. The Chemokine Receptor CXCR3 Is Degraded following Internalization and Is Replenished at the Cell Surface by De Novo Synthesis of Receptor. *J Immunol* (2008) **180**:6713–6724. doi:10.4049/jimmunol.180.10.6713

225. Brady J, Carotta S, Thong RPL, Chan CJ, Hayakawa Y, Smyth MJ, Nutt SL. The Interactions of Multiple Cytokines Control NK Cell Maturation. *J Immunol* (2010) **185**:6679–6688. doi:10.4049/jimmunol.0903354

226. Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei X, Liew FY, Caligiuri MA, Durbin JE, Biron CA. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* (2002) **169**:4279–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12370359 [Accessed December 3, 2018]

227. Balashov KE, Rottman JB, Weiner HL, Hancock WW, Talmage DW. CCR5+ and CXCR3+ T cells are increased in multiple sclerosis and their ligands MIP-1-alpha and IP-10 are expressed in demyelinating brain lesions. *Immunology* (1999) **96**:6873–6878. doi:10.1073/pnas.96.12.6873

228. Trifilo MJ, Montalto-Morrison C, Stiles LN, Hurst KR, Hardison JL, Manning JE, Masters PS, Lane TE. CXC chemokine ligand 10 controls viral infection in the central nervous system: evidence for a role in innate immune response through recruitment and activation of natural killer cells. *J Virol* (2004) **78**:585–94. doi:10.1128/JVI.78.2.585-594.2004

229. Bernink JH, Krabbendam L, Germar K. Interleukin-12 and-23 Control Plasticity of CD127 + Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. (2015) doi:10.1016/j.immuni.2015.06.019

230. Pont S. Thy-1: a lymphoid cell subset marker capable of delivering an activation signal to mouse T lymphocytes. *Biochimie* (1987) **69**:315–320. doi:10.1016/0300-9084(87)90022-8

231. Haeryfar SMM, Hoskin DW. Thy-1: More than a Mouse Pan-T Cell Marker. *J Immunol* (2004) **173**:3581–3588. doi:10.4049/jimmunol.173.6.3581

232. Gorczynski RM. CD200:CD200R-Mediated Regulation of Immunity. *ISRN Immunol* (2012) **2012**:1–18. doi:10.5402/2012/682168

233. Walker DG, Lue L-F. Understanding the neurobiology of CD200 and the CD200 receptor: a therapeutic target for controlling inflammation in human brains? *Future Neurol* (2013) **8**: doi:10.2217/fnl.13.14

234. Wright GJ, Puklavec MJ, Willis AC, Hoek RM, Sedgwick JD, Brown MH, Barclay AN.

Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. *Immunity* (2000) **13**:233–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10981966 [Accessed December 5, 2018]

235. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, Blom B, Homola ME, Streit WJ, Brown MH, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science* (2000) **290**:1768–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11099416 [Accessed December 5, 2018]

236. Chitnis T, Imitola J, Wang Y, Elyaman W, Chawla P, Sharuk M, Raddassi K, Bronson RT, Khoury SJ. Elevated Neuronal Expression of CD200 Protects Wlds Mice from Inflammation-Mediated Neurodegeneration. *Am J Pathol* (2007) **170**:1695–1712. doi:10.2353/ajpath.2007.060677

237. Meuth SG, Simon OJ, Grimm A, Melzer N, Herrmann AM, Spitzer P, Landgraf P, Wiendl H. CNS inflammation and neuronal degeneration is aggravated by impaired CD200–CD200R-mediated macrophage silencing. *J Neuroimmunol* (2008) **194**:62–69. doi:10.1016/J.JNEUROIM.2007.11.013

238. Liu Y, Bando Y, Vargas-Lowy D, Elyaman W, Khoury SJ, Huang T, Reif K, Chitnis T. CD200R1 agonist attenuates mechanisms of chronic disease in a murine model of multiple sclerosis. *J Neurosci* (2010) **30**:2025–2038. doi:10.1523/JNEUROSCI.4272-09.2010

239. Robinette ML, Fuchs A, Cortez VS, Lee JS, Wang Y, Durum SK, Gilfillan S, Colonna M. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat Immunol* (2015) **1**:45–46. doi:10.1038/ni.3094

240. Marquardt N, Béziat V, Nyström S, Hengst J, Ivarsson MA, Kekäläinen E, Johansson H, Mjösberg J, Westgren M, Lankisch TO, et al. Cutting edge: identification and characterization of human intrahepatic CD49a+ NK cells. *J Immunol* (2015) **194**:2467–71. doi:10.4049/jimmunol.1402756

241. Aktas O, Smorodchenko A, Brocke S, Infante-Duarte C, Topphoff US, Vogt J, Prozorovski T, Meier S, Osmanova V, Pohl E, et al. Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL. *Neuron* (2005) **46**:421–432. doi:10.1016/j.neuron.2005.03.018

242. Hilliard B, Wilmen A, Seidel C, Liu T-STS, Göke R, Chen Y, Goke R, Chen Y. Roles of TNF-Related Apoptosis-Inducing Ligand in Experimental Autoimmune Encephalomyelitis. *J Immunol* (2001) **166**:1314–1319. doi:10.4049/jimmunol.166.2.1314

243. Ikeda T, Hirata S, Fukushima S, Matsunaga Y, Ito T, Uchino M, Nishimura Y, Senju S. Dual effects of TRAIL in suppression of autoimmunity: the inhibition of Th1 cells and the promotion of regulatory T cells. *J Immunol* (2010) **185**:5259–5267. doi:10.4049/jimmunol.0902797

244. Lünemann JD, Waiczies S, Ehrlich S, Wendling U, Seeger B, Kamradt T, Zipp F. Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells. *J Immunol* (2002) **168**:4881–4888. doi:10.4049/jimmunol.168.10.4881

245. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, Wang Y. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ* (2007) **14**:2076–2084. doi:10.1038/sj.cdd.4402220

246. Nielsen N, Ødum N, Ursø B, Lanier LL, Spee P. Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. *PLoS One* (2012) **7**:e31959. doi:10.1371/journal.pone.0031959

247. Schuster IS, Wikstrom ME, Brizard G, Coudert JD, Estcourt MJ, Manzur M, O'Reilly LA, Smyth MJ, Trapani JA, Hill GR, et al. TRAIL+ NK Cells Control CD4+ T Cell Responses during Chronic Viral Infection to Limit Autoimmunity. *Immunity* (2014) **41**:646–656. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1074761314003471 [Accessed April 18, 2018]

248. Tecchio C, Huber V, Scapini P, Calzetti F, Margotto D, Todeschini G, Pilla L, Martinelli G, Pizzolo G, Rivoltini L, et al. IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. *Blood* (2004) **103**:3837–44. doi:10.1182/blood-2003-08-2806

249. Mariani SM, Krammer PH. Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol* (1998) **28**:973–982. doi:10.1002/(SICI)1521-4141(199803)28:03<973::AID-IMMU973>3.0.CO;2-T

250. Schneider-Brachert W, Heigl U, Ehrenschwender M. Membrane Trafficking of Death Receptors: Implications on Signalling. *Int J Mol Sci* (2013) **14**:14475–14503. doi:10.3390/ijms140714475

251. Ludwig A, Schulte A, Schnack C, Hundhausen C, Reiss K, Brodway N, Held-Feindt J, Mentlein R. Enhanced expression and shedding of the transmembrane chemokine CXCL16 by reactive astrocytes and glioma cells. *J Neurochem* (2005) **93**:1293–1303. doi:10.1111/j.1471-4159.2005.03123.x

252. Rosito M, Deflorio C, Limatola C, Trettel F. CXCL16 orchestrates adenosine A3 receptor and MCP-1/CCL2 activity to protect neurons from excitotoxic cell death in the CNS. *J Neurosci* (2012) **32**:3154–63. doi:10.1523/JNEUROSCI.4046-11.2012

253. le Blanc LMP, van Lieshout AWT, Adema GJ, van Riel PLCM, Verbeek MM, Radstake TRDJ. CXCL16 is elevated in the cerebrospinal fluid versus serum and in inflammatory conditions with suspected and proved central nervous system involvement. *Neurosci Lett* (2006) **397**:145–148. doi:10.1016/j.neulet.2005.12.029

254. Rosito M, Lauro C, Chece G, Porzia A, Monaco L, Mainiero F, Catalano M, Limatola C, Trettel F. Trasmembrane chemokines CX3CL1 and CXCL16 drive interplay between neurons, microglia and astrocytes to counteract pMCAO and excitotoxic neuronal death. *Front Cell Neurosci* (2014) **8**:193. doi:10.3389/fncel.2014.00193

255. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell– and B cell–independent adaptive immunity mediated by natural killer cells. *Nat Immunol* (2006) **7**:507–516. doi:10.1038/ni1332

256. Cortez VS, Ulland TK, Cervantes-Barragan L, Bando JK, Robinette ML, Wang Q, White AJ, Gilfillan S, Cella M, Colonna M. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. *Nat Immunol* (2017) **18**:995–1003. doi:10.1038/ni.3809

257. Keskin DB, Allan DSJ, Rybalov B, Andzelm MM, Stern JNH, Kopcow HD, Koopman LA, Strominger JL. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci* (2007) **104**:3378–3383. doi:10.1073/pnas.0611098104

258. De Groot CJ, Montagne L, Barten AD, Sminia P, Van Der Valk P. Expression of transforming growth factor (TGF)-beta1, -beta2, and -beta3 isoforms and TGF-beta type I and type II receptors in multiple sclerosis lesions and human adult astrocyte cultures. *J Neuropathol Exp Neurol* (1999) **58**:174–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10029100 [Accessed December 8, 2018]

259. Luo J, Ho PP, Buckwalter MS, Hsu T, Lee LY, Zhang H, Kim D-KK, Kim S-JJ, Gambhir SS, Steinman L, et al. Glia-dependent TGF-β signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis. *J Clin Invest* (2007) **117**:3306–3315. doi:10.1172/JCI31763

260. Lanz T V., Ding Z, Ho PP, Luo J, Agrawal AN, Srinagesh H, Axtell R, Zhang H, Platten M, Wyss-Coray T, et al. Angiotensin II sustains brain inflammation in mice via TGF-beta. *J Clin Invest* (2010) **120**:2782–2794. doi:10.1172/JCI41709.2782

261. Peng H, Jiang X, Chen Y, Sojka DK, Wei H, Gao X, Sun R, Yokoyama WM, Tian Z. Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. *J Clin Invest* (2013) **123**:1444–1456. doi:10.1172/JCI66381

262. Wang XX, Peng H, Cong J, Wang XX, Lian Z, Wei H, Sun R, Tian Z. Memory formation and long-term maintenance of IL-7Rα+ ILC1s via a lymph node-liver axis. *Nat Commun* (2018) **9**:4854. doi:10.1038/s41467-018-07405-5

263. Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, Wu PM, Doykan CE, et al. Identification of a unique TGF-β–dependent molecular and functional signature in microglia. *Nat Neurosci* (2014) **17**:131–143. doi:10.1038/nn.3599

264. Garofalo S, D'Alessandro G, Chece G, Brau F, Maggi L, Rosa A, Porzia A, Mainiero F, Esposito V, Lauro C, et al. Enriched environment reduces glioma growth through immune and non-immune mechanisms in mice. *Nat Commun* (2015) **6**: doi:10.1038/ncomms7623

265. Quatrini L, Wieduwild E, Guia S, Bernat C, Glaichenhaus N, Vivier E, Ugolini S. Host resistance to endotoxic shock requires the neuroendocrine regulation of group 1 innate lymphoid cells. *J Exp Med* (2017) **214**:3531–3541. doi:10.1084/jem.20171048

266. Jiang W, Li D, Han R, Zhang C, Jin W-N, Wood K, Liu Q, Shi F-D, Hao J. Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages. *Proc Natl Acad Sci U S A* (2017) **114**:E6202–E6211. doi:10.1073/pnas.1705491114

9 Selbständigkeitserklärung

Ich, Silvina Romero Suárez, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: “Migrant or Resident? The identification of group 1 ILCs in the murine CNS “ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die Hilfe Dritter verfasstund auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.